Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 107 JUNE 1961 No. 2

SECTION MEETINGS

ILLINOIS
University of Illinois Medical School May 9, 1961

Onset of Follicular Atresia Following Hypophysectomy of the Laying Hen.* (26587)

H. OPEL† AND A. V. NALBANDOV
Department of Animal Science, University of Illinois, Urbana

In the laying hen hypophysectomy results in rapid degeneration of all ovarian follicles which have entered the final stage of greatly accelerated growth. Atresia may be macroscopically visible in such follicles as early as 12 hours after pituitary removal(1).

A recent and unexpected observation in this laboratory has indicated that of the 8 to 14 rapidly developing follicles usually found in the ovary of a laying hen, those of intermediate size (5-10 g) are most resistant to atresia after hypophysectomy and that they remain ovulable for several hours after collapse of the mature follicle(2). In the present report the relationship of follicle size to onset of atresia is further examined.

Materials and methods. Thirty-two regularly laying White Leghorn hens, kept in individual cages and subjected to a constant 14-hour-light day, were hypophysectomized midpoint in a clutch of 4 or more eggs.

The birds were killed for examination of the ovaries at intervals of 6 to 72 hours after operation. All follicles in the final stage of accelerated growth (0.09 g or larger) were removed from the ovaries, weighed and examined individually for macroscopically visible signs of atresia. The follicles of each ovary were divided into 5 classes according to size, and percent of atretic follicles in each class was calculated.

Results. The results establish conclusively that follicles of intermediate size are the most resistant to degeneration following pituitary removal (Table I, Fig. 2). Atresia was first detected in the smallest follicles, but by 18 hours after hypophysectomy mature follicles (15 or more g) were the most heavily affected. By 24 hours all follicles in 4 out of 6 hens were completely atretic. In the remaining birds there were one or 2 follicles in the 5- to

* This study was supported, in part by grants from USPHS and the USDA. The interest of Dr. R. M. Fraps is gratefully acknowledged.

† Present address: Agricultural Research Service, U. S. Department of Agric., Beltsville, Md.

TABLE I. Effect of Hypophysectomy on Rate of Follicular Atresia of Laying Hens.

Hr after operation	No. of hens	% follicles atretic in weight class (g)					
		15+	10–14.9	5–9.9	1–4.9	.4–.99	.09–.39
6	6	0	0	0	0	0	19.7
12	6	50.0	10.0	20.0	1.2	0	47.5
18	6	100.0	57.0	20.0	0	0	60.7
24	6	100.0	100.0	67.0	100.0	100.0	100.0
48	3	100.0	100.0	100.0	100.0	100.0	100.0
72	3	100.0	100.0	100.0	100.0	100.0	100.0

10-g class which were still intact. The rapid collapse and resorption of the ovarian follicles is shown in Figs. 1, 2, 3, and 4.

Discussion. On the basis of size of follicles, their probable growth rate and their assumed needs for hormonal support, it would have seemed logical to postulate that the largest follicles should have become atretic first, followed by those of lesser size and ending with atresia of the smallest ones. There is no obvious explanation for the unexpected relationship between follicular size and the order in which atresia affects them. Since rate of follicular growth, or more specifically rate of deposition of yolk components at the surface of the growing ovum, is not uniform throughout the period of 7 to 10 days required for the rapidly developing follicle to attain maximum size(3), the possibility arises that intensity of metabolic activity within a follicle at time of hypophysectomy may determine the speed of post-operative regression. Present knowledge of fluctuations in metabolic activity during the accelerated growth period, however, does not fully support such a correlation.

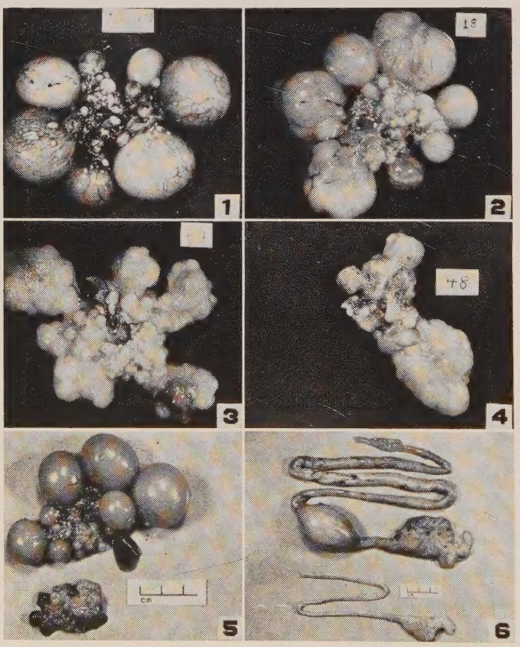


FIG. 1. Ovary of normally laying hen.

FIG. 2, 3, 4. Effect of hypophysectomy 18, 24 and 48 hr after operation. Note in Fig. 2 that the largest and smallest follicles are atretic while medium sized ones are not.

Fig. 5,6. Comparison of ovaries and oviducts of normal hen and hypophysectomized hen 6 days after operation.

Smith(4) has recently shown that rate of follicular growth (percent change in weight per hour) is maximum in follicles entering the phase of accelerated growth and decreases in an approximately linear fashion as follicle size increases. Permeability of the follicle wall, which presumably regulates the transfer of yolk materials to the ovum, follows a similar pattern of change. Using the rate of transfer of Evans' blue dye from the blood to yolk as a third measure of metabolic activity, Smith interprets his results to mean that the transfer rate increases to a maximum in follicles weighing 2-3 g, then decreases somewhat linearly with increasing size. These methods give, at best, an approximation of metabolic activity of follicles. With these facts in mind, the present results indicate that follicles exhibiting the greatest metabolic activity are the first to show signs of post-operative degeneration. However, onset of atresia in these follicles (0.09-0.39 g) is highly variable and roughly 40% of them are still intact 14 hours after hypophysectomy. Mature follicles, which according to Smith's standards are the least active metabolically, are the next group to show a high percentage of atresia. Once initiated, atresia rapidly spreads to all follicles in this class, and by the eighteenth hour all mature follicles have reached an advanced state of regression. Finally, the follicles which are

most resistant to atresia (5-10 g) had been developing at an intermediate and slowly declining rate which shows no sharp demarcation from the growth rate of follicles in adjoining weight classes.

Summary. Ovarian follicles in the laying hen were found to exhibit a differential rate of atresia following hypophysectomy which is dependent on follicle size. Atresia appeared first in the smallest of the rapidly developing follicles at 6 hours post-operatively and spread to include all mature or nearly mature follicles by 18 hours. Follicles weighing 5 to 10 g remained intact for as long as 24 hours.

1. Rothchild, I., Fraps, R. M., *J. Clin. Endocrinol.*, 1948, v8, 615.

2. Opel, H., Nalbandov, A. V., *Endocrinol.*, 1961, in press.

3. Warren, D. C., Conrad, R. M., *J. Agri. Res.*, 1939, v58, 875.

4. Smith, A. H., *Poultry Sci.*, 1959, v38, 1437.

Received March 15, 1961. P.S.E.B.M., 1961, v107.

A Thermostable Cytotoxic Factor in Normal Human Serum Active Against Landschutz Ascites Tumor Cells.* (26588)

ITZHAK GINSBURG†, THEODOR DISHON, MARIANNE BLOCH AND JACK GROSS‡
Department of Experimental Medicine & Cancer Research, Hebrew University Hadassah Medical School, Jerusalem, Israel

During previous studies on the cytotoxicity of various agents on Landschutz ascites cells *in vitro*(1,2,3) it was found that fresh normal human serum (NHS) showed a marked cytotoxic effect. Bolande and Todd(4) have reported a similar cytotoxic effect on HeLa cells and Willheim *et al.*(5,6) and Bolande and McClain(7) on Ehrlich ascites cells (EA). According to the latter authors normal human serum contains a thermolabile nondialysable cytotoxic factor active against both Ehrlich cells and Sarcoma 180 cells. They also found that heating to 56°C for 30 minutes and fixation of complement eliminated toxicity for the cells studied.

The present paper reports further information as to the nature of this cytotoxic factor (e.g. that it is stable to 56°C) and as to its mechanism of action (e.g. that it is absorbed by tumor cells and interferes with anti-tumor cell heterologous antibody action).

Materials and methods. Ascites cells (A) were obtained from white mice (local strain) infected with Landschutz ascites tumor. The tumor cells were maintained by weekly passages. Using aseptic precautions, the ascitic fluid was removed 7 days following infection. The cells were washed with Hank's balanced salt solution (BSS) and diluted to a concentration of approximately 5×10^7 cells per ml.

Sera and complement reagents. Pooled normal human serum (NHS) obtained from at least 8 normal donors was stored at –20°C. Serum reagent lacking complement (C′) components C'_1 and C'_2 ($R_1 + R_2$) was prepared by heating NHS at 56°C for 30 minutes which was then designated as NHS 56. Serum reagents lacking properdin (P) $C'_3 + C'_4$ (RP, $R_3 + R_4$) were prepared according to the method described by Kabat and Mayer(8) and Pillemer *et al.*(9). Complement titrations were performed according to the method described by Kabat and Mayer(8) using anti-sheep hemolysin (Difco). C'_{50} unitage of complement was used exclusively.

* Supported in part by the Rose Allen, Irving & Elaine Miller Memorial Club of Chicago, the Florence Kaminsky Cancer Research Club of Chicago, and the Ann Seifer Memorial Club for Cancer Research of Chicago. Presented in part at Symposium on Mammary Cancer Problems, Tel Aviv, Oct. 25-26, 1960.

† At present Magnes Fellow, Research Dept., Children's Hosp., Philadelphia.

‡ This work was carried out during an assignment of one of the authors (JG) as World Health Organization visiting Professor to Hebrew Univ. Hadassah Medical School in collaboration with members of the national staff.

Normal guinea pig (NGPS) and normal rabbit serum (NRS) were pooled from at least 4 animals. The sera were stored at −20°C. In some experiments C′ reagents were prepared from NRS according to the methods mentioned above.

Cytotoxic studies. Washed cells (5×10^6/ml) together with various sera or C′ reagents were made up to a total volume of 1 ml with BSS. The cell suspension was then incubated for 30 minutes at 37°C. At the end of incubation period, 200 cells were counted in each case and the percentage of tumor cells stained with 1% trypan-blue estimated.

Cell suspensions were prepared from the following tissues: Normal human liver, kidney, heart, placenta, amnion, R-III-mouse tumor, (MMClA mammary Ca(14)). The tissue fragments were thoroughly washed with cold BSS, cut into pieces of approximately 1×1 mm with scissors and forced through a metal sieve of 80 mesh. The suspensions were centrifuged at 500 rpm for one minute and the supernatant discarded. The sedimented cells were washed twice with 20 ml of BSS and finally diluted to contain 5×10^7 cells per ml. Such cell suspensions usually contained over 50% of dead cells. In some experiments cell suspensions were also prepared from 7 day old cultures of HeLa (Gey) and Cang normal liver cells. The cells were removed from the culture vessel by 0.02% Ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) deficient in Ca^{++} and Mg^{++}; 5×10^6 cells per ml were prepared in BSS.

Fractionation of sera. Serum fractions were prepared by 2 methods, (a) by a modification of Cohn's method(10) and (b) by the Zn-acetate modification of the Cohn technic(11).

Agar electrophoretic studies on the various fractions were performed by a modified method of Aronsson and Gronwald(12) developed in this laboratory using Tris-EDTA-Borate buffer (pH 8.9) applying either 75 V for 16 hours or 130 V for 8 hours. Bromphenol blue was used for visualizing the proteins.

Protein determinations were performed by the tyrosine method of Folin-Ciocalteu(13) using human crystalline albumin as the standard. Readings were performed in a Klett-Summerson colorimeter with filter No. 660.

Anti-Landschutz and anti-HeLa antibodies. Antibodies against ascites cells and HeLa cells were obtained by injecting whole cells in complete Freund's adjuvant subcutaneously into rabbits. Ten days after the second injection, the rabbits were bled from the marginal ear vein and the serum titrated against the cells for cytotoxic activity. A titer of 1:30/ml for 100% death was found, and both cell types underwent rapid cytotoxic changes when brought into contact with their respective antibody. The reactions were complement-dependent, as the antisera heated to 56°C lost their cytotoxic activity. This activity could be restored however by addition of NGPS.

Results. Cytotoxic effect of NHS on ascites cells. The cytotoxic activity of NHS, NHS 56, as well as that of various serum fractions on ascites cells is shown in Fig. 1. The cytotoxic activity of NHS can be destroyed by heating to 56°C for 30 minutes (NHS 56). It was also found that cytotoxic activity of NHS could be prevented by EDTA M/1000, heparin 1 μ/ml, and by streptokinase-activated human fibrinolysin, suggesting that complement-like factors are probably involved in the cytolytic reaction(2,15).

These results corroborate those of Bolande and McClain(7) describing the complement-like nature of the cytolytic factor in human serum. It was also found (Fig. 1) that full cytotoxic activity of NHS 56 could be restored by addition of NRS—containing as little as 2 C'_{50} units, but NGPS, having 40 C'_{50}, was without effect. On the other hand NRS failed to activate NHS heated to 63°C for 30 minutes.

NHS 56 could be substituted by the beta globulin fraction of NHS, but not by the Cohn alpha or gamma globulin fractions. NRS heated to 56°C for 30 minutes, failed to activate NHS 56 or beta globulin. These results suggest that thermolabile factors, present in both NHS and NRS and a thermostable factor (SF) present in NHS only, are involved in this reaction, and that the labile factors contributed by NRS are specific for activation of SF. The nature of SF was therefore further investigated.

Relation of SF to complement (C′). Bo-

lande and McClain(7) have shown that the cytotoxic property of NHS depends on presence of all 4 C′ components and that destruction of any of the C′ fractions impaired the cytotoxicity greatly.

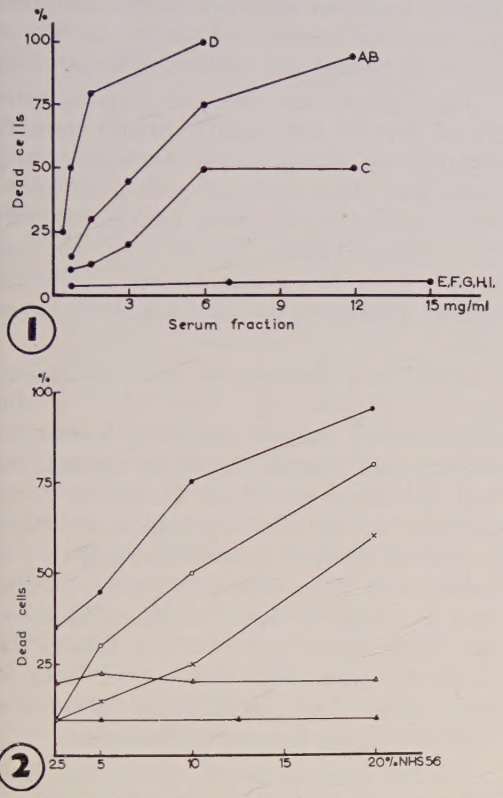


FIG. 1. Effect of normal human serum (NHS) and various serum fractions on Landschutz ascites cells.
A—NHS
B—NHS 56 + NRS 20% ($4C'_{50}$ Hemolytic U.).
C—NHS 56 + NRS 10% ($2C'_{50}$ Hemolytic U.).
D—β Globulin + NRS 20% ($4C'_{50}$ Hemolytic U.).
E—α Globulin + NRS 20% ($4C'_{50}$ Hemolytic U.).
F—γ Globulin + NRS 20% ($4C'_{50}$ Hemolytic U.).
G—NHS 56 + NGPS 20% ($20C'_{50}$ Hemolytic U.).
H—Albumin + NRS 20%.
I —NRS.
Serum and serum fractions incubated with A cells for 30 min. at 37°C prior to reading.

FIG. 2. Absorption of NHS 56 on cells.
●——● NHS 56 Control.
NHS 56 absorbed with:
Chang (10^6 cells).
Kidney suspension.
Liver (5×10^8 cells).
Heart suspension.
○——○ Placenta, amnion.
×——× HeLa (3.5×10^5 cells).
△——△ RIII (2×10^6 cells).
▲——▲ Landschutz ascites (5×10^6 cells).
NHS 56 incubated with cell suspensions for 30 min. at 37°C, and supernate assayed for cytotoxic activity on ascites cells in presence of NRS.

TABLE I. Activation of Human Complement Components by NRS.

Reaction mixture	% dead cells*
Ascites cells + NHS 10%†	95
" + NHS 56 10%	3
" + NHS 56 + NRS 20%	95
" + NRS 20%	3
" + NHS 56 R_3 10%	3
" + NHS 56 R_4 10%	3
" + NHS 56 R_3 10% + NRS 20%	80
" + NHS 56 R_4 10% + NRS 20%	25
" + NHS 56 R_3 10% + NHS 56 R_4 10% + NRS 20%	95
" + NHS 56 10% + NHS R_3 10% + NHS R_4 10%	95
" + NHS 56 10% + NRS – R_4 10%	3

* Results were read 30 min. after addition of ascites cells.
† Final concentration in system.

Since NRS could activate NHS 56 which contains only C'_3 and C'_4, the role of both these components in the cytotoxic reaction was determined. R_3 and R_4 reagents of C′ were prepared from NHS 56 and employed in cytotoxicity tests in presence of NRS. Table I shows that NRS added to NHS 56 R_3 caused similar cytotoxicity to that obtained with NHS 56. On the other hand, removal of C'_4 (R_4) markedly reduced its cytotoxicity in presence of NRS. This suggests that the SF has properties similar to the C'_4 of complement. Destruction of C'_4 of NRS by NH_4OH (NRS.R_4) resulted in no cytotoxic activity in the NHS 56 system.

Absorption experiments (Table II). When NHS or NHS 56 were incubated with ascites cells, even at 4°C, SF activity of the serum disappeared from the supernate, after removal of the cells by centrifugation. SF activity was however demonstrable on the absorbing cells, since cell death followed addition of NRS. On the other hand NRS incubated with ascites cells retained its full capacity to activate NHS 56 showing that the NRS activator is not absorbable on tumor cells.

Under the same experimental conditions when NHS was absorbed with cells at 4°C the supernatant failed to kill fresh cells or to activate NHS 56. These results would indi-

TABLE II. Effect of Absorption on Cytotoxic Activity of NHS.

Reaction mixture	% dead cells
NHS 56 10% + NRS 20%	70
NHS 56 + A* + NRS	6
NHS 56 + NRS + A†	70

* NHS 56 was incubated at 37°C for 30 min. with 2×10^6/ml ascites cells. Cells were removed by centrifugation and supernatant assayed for residual cytotoxicity.

† Same as (*) employing NRS.

cate that during the absorption process NHS was deprived of C′ components (probably C'_1 and C'_2) since there was activation of NHS 56 by C'_1 and C'_2 (NHS 56 + $NHSR_3$ + R_4, Table I).

This assumption was further investigated in experiments in which NHS or NHS 56 were incubated with immune aggregates (gamma globulin + anti gamma globulin) (16). Table III shows that while immune aggregates completely removed cytotoxic activity from NHS the activity of NHS 56 remained unimpaired, pointing to the role of C'_1 and C'_2 in this process. The difference in activity between NHS and NHS 56 after incubation with immune aggregate is probably due to C_4 destruction in the former (cf. discussion).

Role of cations on SF absorption. The absorbability of SF was not impaired by the presence of EDTA, citrate, or by dialysis of NHS 56 for 36 hours against normal saline containing EDTA or citrate. This shows that metal ions are not essential to this process. However, addition of EDTA to the NHS 56 + NRS system completely abolished the cytotoxic phenomenon, pointing to the fact that cations are involved in activation of the cytotoxic mechanism.

TABLE III. Effect of Complement Fixation on SF.

Reaction mixture	% dead cells
NHS 10%	75
" + immune precipitate* 10%	5
" + immune precipitate + NRS 20%	16
NHS 56 10%	5
" + immune precipitate + NRS	76
" + NRS	90

* BSS — Washed precipitate obtained from gamma globulin-anti gamma globulin rabbit serum.

Affinity of SF to mammalian cells. Bolande *et al.*(4,7) and Fedoroff(17,18,19) have shown that the affinity of the cytotoxic factor in human serum is not confined only to mouse tumors but also to other mammalian cells. We therefore investigated further the affinity of SF to normal and malignant human cells.

Fig. 2 shows that while the cytotoxic activity of NHS 56 was greatly reduced upon absorption on ascites, mouse mammary carcinoma and HeLa cells, no such phenomenon was observed when Chang normal liver cultures, human kidney, liver or heart cells were used. Thus the malignant cells showed a greater affinity to the SF than these normal cells.

Inhibition of heterologous antibodies against ascites and HeLa cells. Effect of SF. Fedoroff(17,18,19) showed that while human serum obtained from schizophrenic patients was toxic to mouse fibroblasts (L-strain) it was not toxic to HeLa cells, grown in a medium containing human serum. Similarly in our experiments HeLa cells, grown either in presence of human or horse serum could absorb the stable factor but were not killed. The possibility that HeLa cells coated with SF would alter their susceptibility to cytotoxic mechanisms was therefore investigated.

Washed suspensions of ascites and HeLa cells were preincubated with SF for 30 minutes at 37°C. The cells were then washed with BSS and both cell suspensions were incubated with the corresponding heterologous antibodies.

System No. 1 contained ascites cells + anti-ascites serum 56 + NGPS. NGPS was employed in this system because it could not activate NHS 56 but supplied the C′ for anti-ascites serum 56.

System No. 2 contained HeLa cells and their corresponding antibody.

Table IV shows that preincubation of both types of cells with SF markedly reduced their susceptibility to heterologous antibody. These experiments suggest that SF blocked specific receptor sites on the cells, formerly susceptible to the heterologous antibody and complement.

Discussion. A thermolabile complement-like cytotoxic factor in human serum has been

TABLE IV. Inhibition of Cytotoxic Antibodies by SF.

Reaction mixture	% dead cells
HeLa (10^6/ml) + 10% rabbit anti-HeLa serum	100
HeLa + NHS 56 20%	5
HeLa treated with NHS 56 for 30′ at 37°C + anti-HeLa 10%	20
HeLa + NRS 20%	5
HeLa treated with NRS 20% for 20′ at 37°C + anti-HeLa 10%	100
Ascites cells (2×10^6/ml) + 10% rabbit anti-ascites serum heated to 56°C + NGPS	100
Ascites cells + 10% NGPS	5
Ascites cells + 10% NHS 56	6
Ascites cells treated with 10% NHS 56 at 37°C for 30′ + anti-ascites serum 10% + 10% NGPS	30

described by various workers. Fedoroff(17,18, 19) has shown that the serum of schizophrenic patients was toxic to mouse fibroblast cells but had no effect on HeLa cells grown in human serum. Bolande and Todd(4) have described the presence of a factor in pooled human sera toxic to fibroblasts (U 12) and HeLa cells grown on horse serum, but innocuous to normal human fibroblast cultures. The complement components to which this activity was ascribed were C'_3 and C'_4 and probably C'_2.

Bolande and McClain(7) report on a heat-labile cytotoxic factor in human serum, against Ehrlich ascites tumor and Sarcoma 180 cells. This factor was absorbed on the cells, with no inactivation of any of the components of complement. Absorption with the U 12 fibroblasts on the other hand was associated with inactivation of some C'_2 and C'_4.

Kuru *et al*:(20) found that NHS 56 can be activated by NRS, but not by normal marmot serum, to cause the death of Ehrlich ascites cells. We have fully corroborated these findings except for their contention that activity of the SF lies in the gamma globulin fraction of the serum. The evidence in our investigation points to the beta globulin fraction as carrying the cytotoxic activity.

Experiments with the different complement components point to the possibility that a C'_4-like component of C′ might be involved in the cytotoxic mechanism. The fact that NHS after absorption on immune aggregates loses its cytotoxic activity (Table III) further substantiates this assumption(16). C'_1 is a proesterase and is the first C′ component which combines with the antigen-antibody complex. As a result of the combination the proenzyme is activated. C'_4 reacts next, followed by C'_2 and they are irreversibly destroyed by the C'_1 esterase(21). As NHS 56 devoid of C'_1 and C'_2 remains active after absorption on immune aggregates, the possibility exists that SF activity might reside with C'_3 or C'_4. Similarly although Mg^{++} and Ca^{++} are not necessary for absorption of the SF on tumor cells, their presence is an absolute requirement for the cytotoxic reaction, (as is the case for complement activity in general).

The role, if any, of the SF serum component in host resistance to tumor remains to be elucidated in the light of the absorption experiments reported here. A reduction in the serum SF contents in cancer patients *vs* normals, observed in preliminary tests, is now being further substantiated.

Note: After submission of this paper for publication the report of Landy *et al.*(22) became available. It appears likely that the factor described therein is closely similar to the SF reported here.

Summary. A heat stable factor (SF) in normal human serum which is cytotoxic to Landschutz ascites tumor cells has been described. The cytotoxic action requires the presence of human or rabbit complement but guinea pig complement is ineffective. The substance is found in the beta-globulin fraction and has characteristics similar to C'_4 of human complement. It is selectively absorbed on human and mouse tumor cells and human placenta but not by normal human liver, kidney or heart. SF inhibits the action of heterologous antibody on ascites and HeLa cells.

We are indebted to Dr. A. Rimon, Marcus Memorial Blood Bank Institute, Jaffa, for the Cohn fractions, and to Dr. Esther Tenenbaum of this Department for a generous supply of HeLa cells and Chang cells.

1. Ginsburg, I. *Brit. J. Exp. Path.,* 1959, v40, 417.
2. Ginsburg, I., Ram, M., *Nature,* 1960, v185, 328.
3. Ginsburg, I., *Brit. J. Exp. Path.,* 1960, v41, 648.

4. Bolande, R. P., Todd, E. W., *A.M.A. Arch. Path.,* 1958, v66, 720.

5. Willheim, R., Icy, H. C., Janacek, H. M., *J. Exp. Med. and Surg.,* 1957, v15, 300.

6. Willheim, R., Revici, E., Fluss, P., Anber, M., *ibid.,* 1959, v17, 272.

7. Bolande, R. P., McClain, J. P., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 345.

8. Kabat, E. A., Mayer, M. M., In *Experimental Immunochemistry,* 1948, Charles C. Thomas Co. Publishers.

9. Pillemer, L., Blum, L., Lepow, I. H., Wurz, L., Todd, E. W., *J. Exp. Med.,* 1956, v103, 1.

10. Lever, W. F., *et al. J. Clin. Invest.,* 1951, v30, 99.

11. *Blood Cells & Plasma proteins,* Editor J. L. Tullis, Academic Press, Inc., 1953.

12. Aronsson, T., Gronwall, A. *Scandinavian J. Clin. and Lab. Invest.,* 1957, v9, 338.

13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. I., *J. Biol. Chem.,* 1951, v193, 265.

14. Pikovski, M., Schlesinger, M., *Cancer Research,* 1959, v19, 222.

15. Mayer, M. M., *Progress in Allergy,* 1958, v5, 215.

16. Lepow, J. H., Pillemer, L., *J. Immunol.,* 1955, v75, 63.

17. Fedoroff, S., *J. Lab. and Clin. Med.,* 1956, v48, 55.

18. ———, *Texas Rep. on Biol. and Med.,* 1958, v1, 32.

19. ———, *J. Exp. Med.,* 1959, v109, 615.

20. Kuru, M., Kosaki, G., Terazawa, T., Itouzi, H., Isibasi, H., Tokunaga, A., *Gann,* 1957, v48, 328.

21. Lepow, I. H., Ratnoff, O. D., Levy, I., *J. Exp. Med.,* 1958, v107, 451.

22. Landy, M., Michael, J. G., Trapani, R. J., Achinstein, B., Woods, M. W., Shear, M., *Cancer Research,* 1960, v20, 1279.

Received January 4, 1961. P.S.E.B.M., 1961, v107.

Effect of Brain Hormone from *Bombyx mori* on Metamorphosis of *Calliphora erythrocephala.** (26589)

MASATOSHI KOBAYASHI AND WALTER J. BURDETTE

Laboratory of Clinical Biology, Department of Surgery, University of Utah College of Medicine, Salt Lake City

Neurosecretion of the brain of insects is known to stimulate the prothoracic gland to function(1-9). Possible direct action of the brain hormone(9) in induction of metamorphosis has been tested in a series of experiments.

Methods.† Mature larvae of *Calliphora erythrocephala*(10) were ligated at the seventh segment and were observed for 24 hr at 25°C. Those with pupation of the segment anterior to the ligature were selected for use in the bioassay. A second ligature was tied posterior to the first and the larva divided between them. After mounting the posterior segment on a glass needle passing through the second ligature, appropriate dilutions of brain hormone and ecdysone, alone and in combination, were injected into respective larval segments. The ligature was useful in closing the anterior end of the specimen after injection and removing it from the needle of the microsyringe. After 24 hours at 25°C, the larval segments were then scored for pupation. Controls included the original bioassay of the brain hormone and ecdysone in which degree of activity was verified in terms of concentration; segments without treatment; and those injected with sesame oil, the solvent for the brain hormone. The results appear in Table I.

Results. Experience with the bioassay indicates that it is qualitative for a single con-

* This investigation was supported by grants from Nat. Inst. Health, Dept. of H.E.W. and Am. Cancer Soc.

† The authors are indebted to Dr. J. Kirimura and Dr. S. Shimizu, Sericultural Experiment Station, Tokyo, for arrangements concerning the preparation of brain hormone. Members of the technical staff of Laboratory of Clinical Biology, University of Utah assisted in preparation of ecdysone, and the technical staff of the Morphological Laboratory, Sericultural Experiment Station participated in preparation of brain hormone.

TABLE I. Effect of Ecdysone and Brain Hormone on Pupation of *Calliphora erythrocephala.*

Injection			Isolated abdomens		
Sesame oil	Ecdysone, γ	Brain hormone, γ	No. pupated	Total No.	% pupated
				60	0
+				20	0
+		38		10	0
+		75		20	0
+		150		20	0
	7		6	20	30
	15		1	10	10
+	29		11	20	55*
+	7	75	14	20	70*
+	15	150	16	20	80*
+	29	150	10	10	100*

* Positive.

centration, and tests are considered positive when 50% or more of the specimens pupate. Dosages of ecdysone of 29 γ yielded positive bioassays; lesser amounts did not. Negative results were obtained when brain hormone up to a dose of 150 γ was injected. However, addition of 75 γ of brain hormone to 7 γ of ecdysone and 150 γ of brain hormone to 15 γ of ecdysone gave positive bioassays despite negative results with these amounts of ecdysone alone. Although not indicated in the Table, control studies were carried out simultaneously with the bioassays.

Ecdysone(11,12) was prepared from pupae of *Bombyx mori* preserved in methanol, and previous bioassay showed the sample of extract used to be active. Brain hormone† was prepared from pupal brains of *Bombyx mori* dissected free without inclusion of corpora cardiaca and corpora allata. Methanol-acetone-ether extraction yielded a crude but active extract. With Dr. Jiro Kirimura, it was found that injected, artificially diapausing, brainless pupae (Dauer-pupae) of the silkworm became imagines in 3 to 5 weeks when the head of each was injected with 0.2 mg of the oily hormone.

Discussion. Williams(5-8) was able to show that a hormone is secreted in the brain of insects which activates the prothoracic gland to secrete hormone which promotes growth and differentiation. This has now been confirmed in *Lepidoptera* by Ichikawa and Nishiitsutsuji(1), Kobayashi(2) and others(13-18). The results of the present work suggest that the brain hormone not only functions in this way but also acts directly on the tissues in conjunction with ecdysone. These 2 pathways of action are illustrated diagrammatically in Fig. 1.

Ichikawa and Nishiitsutsuji-Uwo(19) utilized larvae of the *Eri* silkworm, *Philosamia cynthia ricini,* during the fifth instar and found that 40% of the isolated abdomens became moths following implantation of 3 brains, but whether a subthreshold amount of hormone from prothoracic gland was contained in the isolated abdomens is unknown. Similar uncertainty attaches to the report of Kobayashi and Yamashita(4). They implanted 2 fresh larval brains into the fifth abdominal segment of 61 silkworm larvae at the fifth instar. The abdomen was isolated 2 hours after pupation, and 2 of the 61 animals emerged as moths 34 to 35 days following pupation. This could not be repeated when 10 larval brains were implanted into each of 11 abdomens.

The results obtained by using purified brain hormone and ecdysone suggest that hormonal extracts of the brain have two functions. The first is its tropic function on the prothoracic gland and the second is its direct, synergistic action with ecdysone on induction of metamorphosis. Whether separate brain hormones are responsible for the two functions respectively is a matter of conjecture.

Summary. Appropriate extraction of brains and pupae of *Bombyx mori* yielded active brain hormone and ecdysone respectively. When various concentrations of these 2 hormones were introduced alone and in combination into isolated larval abdomens of *Calliphora erythrocephala,* pupation occurred

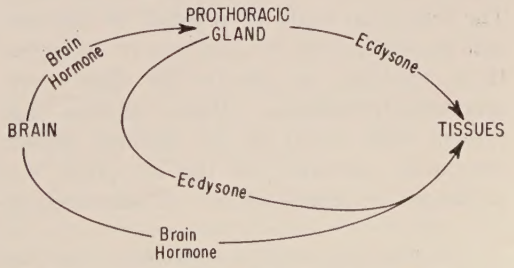


FIG. 1.

when brain hormone was added to concentrations of ecdysone which would not induce pupation alone. (Pupation was not induced by brain hormone alone.) Brain hormone therefore may have a direct action on the tissues when acting synergistically with acdysone in addition to its tropic action on the prothoracic gland.

1. Ichikawa, M., Nishiitsutsuji, J., *Annot. Zool. Japon,* 1951, v24, 205.
2. Kobayashi, M., *J. Sericul. Sci. Japan,* 1955, v24, 389.
3. ———, *Bull. Sericul. Exp. Station,* 1957, v15, 181.
4. Kobayashi, M., Yamashita, Y., *ibid.,* 1959, v28, 67.
5. Williams, C. M., *Biol. Bull.,* 1946, v90, 234.
6. ———, *ibid.,* 1947, v93, 89.
7. ———, *Harvey Lectures,* 1952, Ser. 47, 126.
8. ———, *Biol. Bull.,* 1952, v103, 120.
9. Kobayashi, M., Kirimura, J., *Nature,* 1958, v181, 1217.
10. Fraenkel, G., *Proc. Roy. Soc., London,* 1935, Ser. B, v118, 1.
11. Burdette, W. J., *Acta Union Internat. Contre le Cancer,* 1960, v16, 157.
12. Butenandt, A., Karlson, P., *Z. Naturforschg.,* 1954, v9, 389.
13. Fukuda, S., *Jap. J. Endocrinol.,* 1954, v1, 1.
14. Yashika, K., *Zool. Mag.,* 1954, v63, 363.
15. Nagata, S., Kaji, S., *ibid.,* 1955, v64, 254.
16. Monro, J., *Nature,* 1956, v178, 213.
17. Fukaya, M., *Jap. J. Appl. Zool.,* 1955, v20, 179.
18. Ichikawa, M., Yashika, K., Nishiitsutsuji, J., *Memo. Coll. Sci. Univ. Kyoto,* 1953, Ser. B, v20, 145.
19. Ichikawa, M., Nishiitsutsuji-Uwo, J., *ibid.,* 1960, Ser. B, v27, 9.

Received March 6, 1961. P.S.E.B.M., 1961, v107.

Inhibition of Growth of Streptococci by Adenine.* (26590)

JIMMIE R. NELSON AND ALAN G. C. WHITE
Department of Biochemistry, Tulane University School of Medicine, New Orleans, La.

Bernheimer *et al.*(1) described a synthetic medium for cultivation of *Streptococcus pyogenes,* specifically strain C203S. For several years we have used this medium with success for cultivation of strains C203 and C203S. Recently, however, we experienced difficulty in growing 2 other strains of *S. pyogenes* (S23 and C203U) in the same medium and had occasion to delete adenine and uracil from the medium. The 2 strains which had earlier failed to grow now showed growth comparable to that of strain C203S. On further testing it appeared that the inhibition of strains S23 and C203U was due to adenine. The inhibition could be reversed in the normal growth period by addition of hypoxanthine, xanthine or guanine or their corresponding nucleotides. When adenine was present with uracil in the medium growth eventually occurred but the lag phase was prolonged to several hours. A somewhat similar phenomenon was observed by Pierce and Loring(2) using a pyrimidine deficient mutant of *Neurospora crassa.* The growth of this organism could be inhibited by adenine and yeast adenylic acid. In this case however the reversal of inhibition was brought about by the pyrimidines cytidine or uridine. They further found that guanine caused a similar though less marked inhibition of growth of the mold.

Strain C203U, which is inhibited by adenine, is a mutant of strain C203S which is not. The only reported difference between the 2 strains is that C203U fails to produce streptolysin-S (Bernheimer)(3).

Methods. The organisms used were strains C203S, C203U, S94, and S23 of *S. pyogenes.* These organisms were kindly supplied by Dr. Cornelia A. Eddy, Dept. of Microbiology, Tulane Univ. School of Medicine. The bacterial stock cultures were carried on Pia egg slants at 4°C, transferred monthly. All inocula were prepared in heart infusion broth incubated at 37°C. Incubation time varied

* This work was supported in part by a grant from Louisiana Heart Assn., New Orleans.

TABLE I. Effect of Adenine Concentration on Growth of *S. pyogenes* (C203U).

Adenine conc., μg/ml	O.D. of culture (20 hr)
0 (control)	.54
.1	.62
.5	.51
1	.46
5	.09
10	.10
50	.11

from 8 to 10 hours. The inoculum after incubation was centrifuged, washed twice with physiological saline and diluted with saline to an optical density of approximately 0.4, on a B and L "Spectronic 20" photometer. One drop of this suspension was used as inoculum for 10 ml of the test medium. The synthetic medium was prepared according to the procedure of Bernheimer *et al.*(1) using Bacto-Casamino acids as a source of amino acids. Adenine was deleted from the medium. The original medium was supplemented with inositol, 14 mg per liter and PABA, 10 mg per liter.

Additions to the medium in these experiments were sterilized separately. Ten milliliters of medium were used in each assay tube and each assay was carried out in triplicate. The results presented represent the average growth, in terms of optical density, of the triplicate analysis. In any case where the tubes showed deviation greater than 10% from the average the determination was repeated.

Results. To determine more quantitatively the amount of adenine causing the inhibition, various concentrations of adenine were added to the basal medium with the results noted in Table I.

TABLE II. Reversal of Adenine Inhibition by Yeast Nucleic Acid.

Yeast nucleic acid, μg/ml	O.D. of culture (20 hr)
No adenine, no Y.N.A.	.56
5	.10
10	.11
50	.22
100	.31

All tubes but controls contained 10 μg adenine/ml.

Based upon the earlier experiments of Pierce and Loring(2) we assumed that perhaps an analog inhibition type of phenomenon was occurring. A hydrolysate of yeast nucleic acid was therefore tested. The results are given in Table II. This shows a partial effect of the whole nucleic acid.

By paper electrophoresis of the above hydrolsate (0.01 M PO_4-buffer pH 7.0, for 3 hr at 400 Volts in a Spinco Model R electrophoresis cell) and elution of the ultraviolet quenching areas of the strip it was found that the active materials migrated similarly to known samples of hypoxanthine and xanthine. These materials were therefore tested along with guanine and uric acid (Table III). The inability of uric acid to reverse the inhibition is perhaps understandable since it is an end product of purine metabolism and is not converted metabolically to the purine bases.

TABLE III. Reversal of Adenine Inhibition by Other Purines.

Addition compound		O.D. of culture (20 hr)
No addition		.62
Adenine only		.09
Xanthine	(25 μg/ml)	.70
Hypoxanthine	(" ")	.68
Guanine	(" ")	.58
Uric acid	(" ")	.10

All tubes but controls contained 10 μg adenine/ml.

TABLE IV. Reversal of Adenine Inhibition by Xanthine.

Xanthine, μg/ml	O.D. of culture (20 hr)
No adenine, no xanthine	.77
0	.08
1	.09
2	.27
3.1	.66
6.2	.69
12.5	.77
25	.78
50	.77

All tubes but controls contained 10 μg adenine/ml.
Inhibition ratio approx. 3:1.

The inhibition to antagonist ratio is shown for adenine and xanthine in Table IV. Experiments with the other 2 purines gave essentially the same results. These values are

TABLE V. Effect of Adenine on Growth of 4 Strains of *S. pyogenes.*

Strains	O.D. of culture (20 hr)	Strains	O.D. of culture (20 hr)
C203U (A)	.66	S94 (A)	.82
" (B)	.07	" (B)	.88
" (C)	.54	" (C)	.68
C203S (A)	.97	S23 (A)	.61
" (B)	.92	" (B)	.08
" (C)	.96	" (C)	.45

(A)—Basal without adenine. (B)—Basal + adenine (5 μg/ml). (C)—Basal + adenine (5 μg/ml) + xanthine (2.5 μg/ml).

rather unusual in that the inhibition can be overcome by approximately one-third the molar concentration of antagonist.

To determine whether the growth inhibition by adenine was a more general phenomenon, not limited solely to strain S23, we tested 3 other strains, C203U, C203S, and S94, with the results shown in Table V. It is apparent that 2 of the 4 strains are inhibited by adenine and the inhibition, in both cases, is overcome by xanthine.

Using the same assay procedures we have further demonstrated that adenylic acid in approximately the same concentration as adenine causes inhibition of growth. The inhibition is also reversed by the nucleotides of the 3 purines previously shown to be antagonists to adenine. Whether the cells are able to cleave the nucleotides to the free bases, and whether the free bases are the specific functioning moieties as inhibitor and antagonist, we do not know.

Discussion. There have been few reports in the literature of inhibition of microbial growth by adenine. In none of these has the inhibition been reversed by other purines, rather the general effect described has been that reported by Pierce and Loring(2) that all the purines have a similar inhibitory effect. Recently Levin and Magasanik(4) have demonstrated the repression, by adenine, of inosinicase synthesis in a non-purine requiring strains of *Salmonella typhimurium* and *Aerobacter aerogenes.* On the basis of this observation, the following hypothesis is offered as a possible explanation of our results. Adenine may suppress the formation or inhibit the activity of an enzyme in the common pathway of purine synthesis in a manner comparable to the reported effect of adenine on inosinicase. Addition of other purines which are metabolized and inter-converted beyond the block may overcome this effect by permitting a continuation of nucleic acid synthesis, thus removing the adenine and consequently the inhibitor. We have no experimental evidence for this but since our strain of *S. pyogenes* does not require purines for growth it presumably has the necessary enzymes for the several known interconversions of the various purines and their corresponding nucleotides. Obviously more work is needed before a more definite statement can be made regarding the specific site of inhibition of growth of these cells by adenine.

Summary. Adenine, at a concentration of 5 μg/ml or above in a synthetic medium, has been shown to inhibit growth of 2 strains of *S. pyogenes,* S23 and C203U. The inhibition can be overcome by addition of xanthine, guanine or hypoxanthine at approximately one-third the molar concentration of the inhibitor.

1. Bernheimer, A. W., Gillman, W., Hottle, G. A., Pappenheimer, A. M., Jr., *J. Bact.,* 1942, v43, 495.

2. Pierce, J. G., Loring, H. S., *J. Biol. Chem.,* 1948, v176, 1131.

3. Bernheimer, A. W., in McCarty, *Streptococcal Infections,* p21, Columbia Univ. Press, 1954.

4. Levin, A. D., Magasanik, B., *J. Biol. Chem.,* 1961, v236, 184.

Received April 14, 1961. P.S.E.B.M., 1961, v107.

A New High-Potency Antidiabetic Sulfonylurea [N-(1-hexahydro-1-azepinyl)-N′-*p*-tolyl-sulfonylurea].* (26591)

W. E. Dulin, H. L. Oster and F. G. McMahon (Introduced by J. C. Stucki)
Departments of Endocrinology and Clinical Research, The Upjohn Co., Kalamazoo, Mich.

Since tolbutamide (Orinase†) was released for clinical use and its efficacy proven in treating maturity onset diabetics, an intense search has been conducted for compounds which offer advantages over tolbutamide. This report describes studies conducted with a new blood sugar lowering sulfonylurea, U-17835 ([N-(1-hexahydro-1-azepinyl)-N′-*p*-tolyl-sulfonylurea]) (Fig. 1), which has been found to be more potent than tolbutamide in rats and humans and better tolerated acutely than tolbutamide in both rats and mice.

Methods. Intact rats. Male rats (Sprague-Dawley) 140-160 g were used, following an overnight fast. They were injected subcutaneously with 100 mg glucose in 0.5 cc saline, immediately prior to oral administration of test compounds in 0.5 cc CMC vehicle(1). Blood was withdrawn 2 hours later from posterior vena cava while rats were under barbiturate‡ anesthesia. Blood sugars were determined by Autotechnicon which is a modification of a method described by Hoffman(2). Potency estimates were made by determining from dose-response curves doses of compounds which produced comparable blood sugar lowering.

Adrenalectomized rats. This method is as previously described(3) and measures ability of compounds to lower blood sugar in fasted adrenalectomized rats (7 days post-operative). The animals were injected subcutaneously with 100 mg glucose in 0.5 cc saline immediately prior to oral administration of compounds. Blood sugars were determined by Autotechnicon on blood taken from posterior vena cava, 2 hours following drug treatment. Potency ratios were determined by a standard USP method(4).

Duration of action—intact rats. Male rats (Sprague-Dawley) 140-160 g were used, following an overnight fast. Compounds were given orally in CMC vehicle (0.5 cc), immediately after a 0-time blood sample was taken. Blood was also obtained at 2, 4, and 6 hours following drug administration. Tail blood was taken by pipette from a razor-blade nick. Prior to bleeding, each animal was subjected to 5 min of 39°C temperature. Rats were maintained at room temperature between bleedings. Sugar was determined by Micro-Autotechnicon method.

Normal Humans. Normal, healthy, male prisoner volunteers, 150-180 lb were used to compare U-17835 with tolbutamide. Following an overnight fast, compounds were given orally as compressed tablets, immediately after a 0-time blood sample was obtained. Fasting continued during study. Blood was withdrawn from antecubital vein, 1, 2, 4, 6, 8, and 10 hr after treatment. Sugars were determined on Autotechnicon. All treatments were given on double-blind basis.

Diabetic humans. A preliminary study was conducted, using 3 diabetic prisoners treated with U-17835 for an 8-wk period. Daily fasting blood sugars, urinalysis, and body weights were obtained, together with urinary glucose estimates before each meal and at bedtime (using Tes-Tape). Complete blood counts were done at weekly intervals. Every 2 weeks, blood urea nitrogen, thymol turbidity, bromsulphalein retention, alkaline phosphatase, serum glutamic oxalacetic transaminase, cephalalin flocculation, and erythrocyte sedimentation rates were performed by the usual clinical laboratory methods. The object of

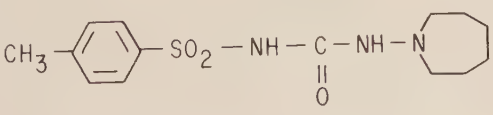
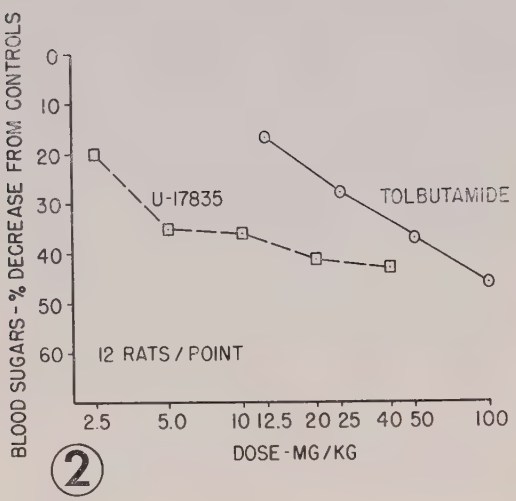


FIG. 1. Structure of U-17835.

* Synthesized and supplied for these studies by Dr. J. B. Wright, Dept. of Chemistry, Upjohn Co. A paper describing the synthesis and properties of this and related compounds will be published later.

† Registered Trademark, Upjohn Co.

‡ 5-(1-Cyclopenten-2-yl)-5-allylbarbituric acid, sodium.



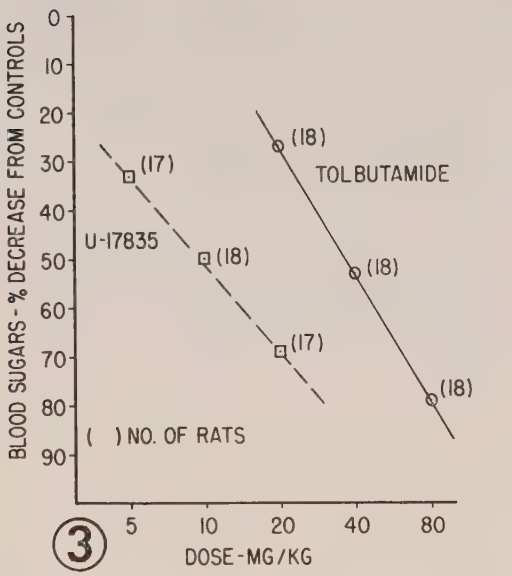


FIG. 2. Comparative blood sugar lowering effects of U-17835 and tolbutamide in glucose-primed, fasted, intact rats, 2 hr after oral administration. Data pooled from 2 experiments.

FIG. 3. Comparative blood sugar lowering effect of U-17835 and tolbutamide in glucose-primed, fasted, adrenalectomized rats, 2 hr after oral administration. Data pooled from 3 separate experiments.

this phase of investigation was to determine clinical efficacy and potency in diabetics, relative to tolbutamide, as well as any obvious side effects or toxicity.

Acute toxicity. LD_{50}'s were determined by intraperitoneal injection in the mouse or oral administration in the rat of a single dose of compound in CMC vehicle. Mice were of mixed sex of the Indianapolis laboratory strain, weighing 20-25 g. Rats were males of the Upjohn strain (Sprague-Dawley ancestry) weighing 150 g. Animals were observed for 7 days following drug. LD_{50} was calculated by method of Spearman-Kärber (5).

Results. Comparison of U-17835 with tolbutamide in intact rats shows that U-17835 was 4-6 times more active than tolbutamide (Fig. 2). A more exact potency determination is not possible since slopes of curves are shallow. In adrenalectomized rats, the ratio of U-17835 to tolbutamide was 4:1 (Fig. 3). This animal preparation gives steeper dose re-

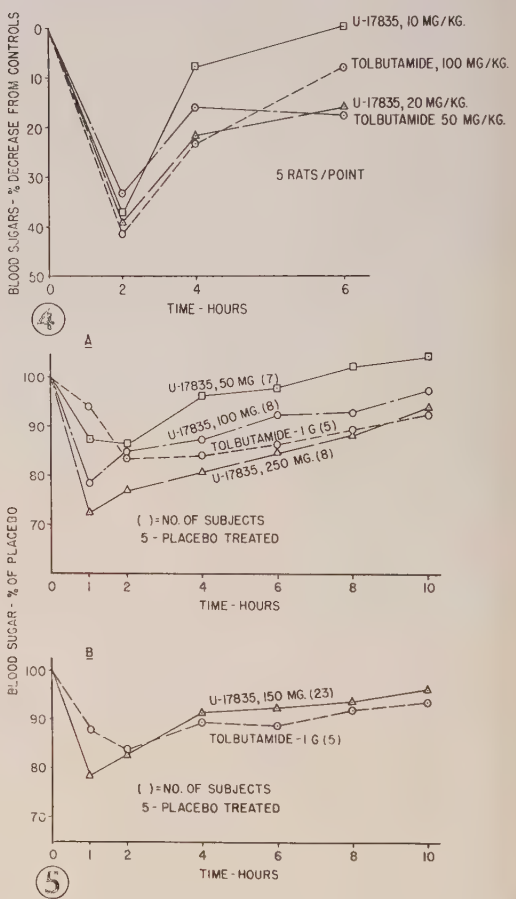


FIG. 4. Comparative duration of hypoglycemic effects of U-17835 and tolbutamide in intact, fasted rats.

FIG. 5. Comparative blood sugar lowering effect of U-17835 and tolbutamide in normal humans (prisoner volunteers) following single doses, administered orally.

TABLE I. Comparative Blood Glucose (Fasting) Responses to U-17835 and Tolbutamide in 3 Diabetics.

Treatment period	Drug	Dose, mg/day	Blood sugar, mg % Avg	(Range)
Pt. A.A.				
1958–59	Tolbutamide	1000	"Normal"	
1959–60	"	500	89	(81–106)
11/19–12/18/60	U-17835	100	90	(80–115)
12/19–12/26/60	"	50	104	(88–126)
12/26– 1/18/61	"	100	84	(70–108)
Pt. H.C.				
1956–1960	NPH insulin	35 units	"Normal"	
11/11–11/19/60	Tolbutamide	2000	96	(68–112)
11/19–11/26/60	U-17835	400	84	(69–109)
11/26–12/ 6/60	"	300	78	(72– 86)
12/ 7–12/13/60	"	100	132	(128–138)
12/22– 1/18/61	"	50	138	(130–146)
12/21– 1/18/61	"	200	85	(70–105)
Pt. A.U.				
1956–1959	PZI*	60 units	"Normal"	
1959–1960	PZI	40 "	88	(71–105)
11/11–11/19/60	Tolbutamide	2000	109	(60–125)
11/19–12/ 5/60	U-17835	400	104	(86–123)
12/ 5–12/12/60	"	200	98	(80–116)
12/13–12/17/60	Placebo	—	148	(106–175)
12/17– 1/15/61	U-17835	200	93	(68–110)

* Protamine zinc insulin.

sponse curves than intact rats, allowing more exact potency calculations. U-17835 was not longer acting than tolbutamide in fasted intact rats (Fig. 4) since blood sugars returned to placebo levels at the same time following equipotent doses.

In normal human subject 150 mg U-17835 was as potent as 1 g tolbutamide (Fig. 5), *i.e.*, U-17835 was about 6-7 times as potent as tolbutamide. These results agree surprisingly well with those obtained in intact rats. No difference in duration of hypoglycemic action of the two drugs is apparent from this study in normal humans.

Three diabetic humans were studied for a 8-wk period (Table I) and it was found that in one (H.C.) 200 mg U-17835 gave control equivalent to that obtained with 2 g tolbutamide. In the second (A.A.) 100 mg U-17835 was equivalent to 0.5 g tolbutamide while in the third (A.U.) 200 mg U-17835 was comparable to 2 g tolbutamide. These results suggest that U-17835 is 5-10 times more effective than tolbutamide in diabetic subjects. No evidence of intolerance or toxicity was observed in these 3 patients.

Oral LD_{50} of U-17835 and tolbutamide in rats was $>$ 5000 mg/kg and 2344 mg/kg, respectively. LD_{50}'s by intraperitoneal injection in mice were 2239 mg/kg for U-17835 and 1288 mg/kg for tolbutamide. Therefore, on a single-dose basis, U-17835 is better tolerated than tolbutamide in mice and rats.

Discussion. Sulfonylureas have been chemically modified in various ways in attempts to achieve some advantage over tolbutamide. Carbutamide and metahexamide are examples of modified sulfonylureas which were more potent but, because they were also more toxic, were unacceptable for clinical use in the U.S.(6,7,8). Chlorpropamide is a more potent sulfonylurea which has been accepted for clinical use in maturity onset diabetics(9). Whereas some of the more active compounds differ structurally from tolbutamide in the aryl portion of the molecule (carbutamide and chlorpropamide), others were modified in both the aryl and alkyl portions (metahexamide). U-17835 is one of the first compounds substituted on only the alkyl portion of the molecule, which exhibits increased potency, and is better tolerated in mice and rats.

West(9) has suggested that true clinical

potency of sulfonylureas in diabetics can be predicted from acute hypoglycemic potency in normals and metabolic half-life. If this is true, then it should be possible to predict half-life from potency in normals and diabetics. Since U-17835 has been found (relative to tolbutamide) to be about as potent acutely in normals as it is clinically in diabetics, it suggests that U-17835 may prove to have a half-life similar to that of tolbutamide.

Although U-17835 may have a half-life similar to tolbutamide, it should be emphasized that duration of action and/or potency can be affected by rates of intestinal absorption. This is supported by observations in other areas of biological activity where pharmaceutical alterations of absorption rates can result in maintenance of effective blood drug levels and increased duration of action(10, 11).

Although U-17835 has a greater potency in man than tolbutamide, extensive clinical trial will be necessary to establish the significance of these findings with U-17835 regarding increased efficacy or fewer side effects compared to tolbutamide.

Summary. U-17835 ([N-(1-hexahydro-1-azepinyl)-N′-*p*-tolyl-sulfonylurea]), a new blood sugar lowering sulfonylurea, was 4-6 times more active than tolbutamide in intact rats and 4 times more active in adrenalectomized rats. At equipotent doses it was not longer acting than tolbutamide in intact rats. Normal human subjects showed U-17835 to be 6-7 times more effective than tolbutamide in lowering blood sugar after a single dose. In 3 diabetic patients, U-17835, given for 8 weeks, was found to be 5-10 times more active than tolbutamide, and it showed no signs of intolerance. This compound was also better tolerated than tolbutamide in both rats and mice.

The authors wish to express their appreciation to P. M. Jones for conducting the acute toxicity studies.

1. Dulin, W. E., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 115.

2. Hoffman, W. S., *J. B. C.,* 1937, v120, 51.

3. Dulin, W. E., Miller, W. L., *Diabetes,* 1959, v8, 199.

4. *Pharmacopeia of U. S.,* 1960, v16, 873.

5. Finney, D. J., *Statistical Methods in Biological Assays.* 1952. Hofner Publishing Co., New York.

6. Drey, N. W., Taussig, K. M., Rubin, B. L., Joshi, R. A., Blumenthal, H. T., *Metabolism,* 1959, v7, 676.

7. Kirtley, W. R., *Diabetes,* 1957, v6, 72.

8. Bradley, R. F., *Ann. N. Y. Acad. Sci.,* 1959, v82, 513.

9. West, K. M., Johnson, P. C., *Diabetes,* 1960, v9, 454.

10. Wagner, J. G., Carpenter, O. S., Collins, E. J., *J. Pharm. and Exp. Therap.,* 1960, v129, 101.

11. *Remington's Practice of Pharmacy.* Editors: Martin, E. W., Cook, E. F., Leuallen, E. E., Osol, A., Tice, L. F., VanMeter, C. T., 1961, Chapt. 38, Mack Publishing Co., Easton, Pa.

Received March 9, 1961. P.S.E.B.M., 1961, v107.

Special Tubes for Total Bile and Pancreatic Juice Collection in the Human.* (26592)

HENRY DOUBILET

Department of Surgery, New York University School of Medicine and Third (New York University) Surgical Division, Bellevue Hospital.

Soft rubber and plastic tubes were designed, which, when implanted in the post-operative wound in patients operated on for biliary and pancreatic disease, permitted physiologically significant measurements of total human bile and pancreatic juice. The rubber tube for bile collection† was constructed like a T-tube, but the long arm was composed of 2 tubes, one draining the liver, the other connected to the lower limb, which was of small

* This study was aided by a grant from Nat. Inst. Health, P.H.S.

† These tubes were manufactured by C. R. Bard, Inc., Summit, N. J.

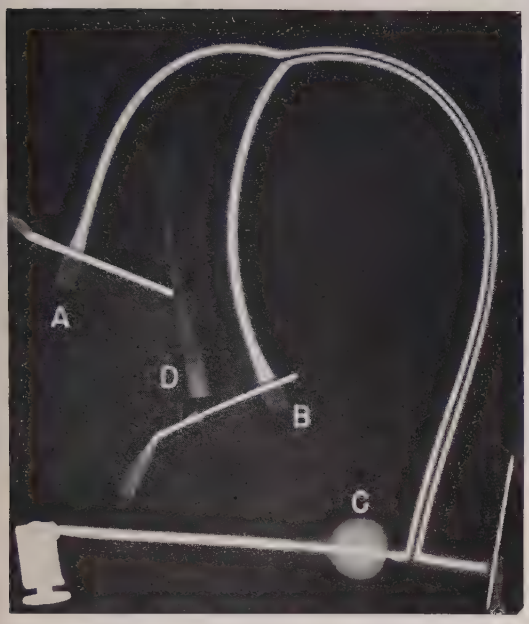


FIG. 1. Special T-tube for total bile collection: X-ray study of Urokon-filled tube to demonstrate its internal structure. Proximal tube (A), distal tube (B), balloon (C), and self-sealing tube to fill balloon (D).

diameter, and extended into the duodenum. Encircling the lower limb was an enlarged, soft balloon which could be inflated with water through a fine tube incorporated into the long arm of the T-tube. This tube was inserted into the common duct in the same manner as an ordinary T-tube. As all studied patients had the sphincter of Oddi sectioned, no hazard was created by the transampullary indwelling tube.

The plastic tube for total collection of pancreatic juice† had a double lumen, a larger lumen draining the pancreatic duct, a finer lumen connected to 2 balloons. One small balloon encircled the terminal end of the tube in the duct of Wirsung, and the other larger balloon was attached at the outside end. The sealed balloons and connecting tube were filled with water, so that compression of the larger accessible outside balloon distended the smaller indwelling balloon. The technic of intubation of the pancreatic duct was described previously (1).

Bile was collected in a plastic bag attached to the proximal tube, and pancreatic juice in a plastic bottle immersed in an ice-filled thermos bowl. Volumes were recorded every 2 hours, a small aliquot kept for analysis, and the rest of the bile or pancreatic juice returned to the patient either through the distal arm of the biliary tube or through a gastrostomy tube if the biliary tract was not drained.

After the tubes described here were perfected, 238 24-hour collections of bile were made on 22 patients, and 75 24-hour collections of pancreatic juice were made on 38 patients. Total collections were started about 12 days after operation, when the patients were up and around and eating 3 regular meals a day. The tubes were tolerated without complaint and removed at will when the experiments were concluded. There were no untoward effects. Occasionally the pancreatic tube slipped out into the duodenum, terminating the collection of pancreatic juice.

The analyses of these juices, and the effect of disease, diet and drugs will be reported elsewhere. However, as indication of the effectiveness of these tubes, it is of interest to note that the 24-hour volume of bile from apparently normal livers, varied from 1000-2000 ml and that the 24-hour volume of pancreatic juice from apparently normal pancreas, varied from 1500-4000 ml in different individuals.

Discussion. The special biliary tube is unique in its application to the biliary tract, in that not only is the total secreted bile collected but also all the bile can be returned to the patient's duodenum at will. Thus, bile

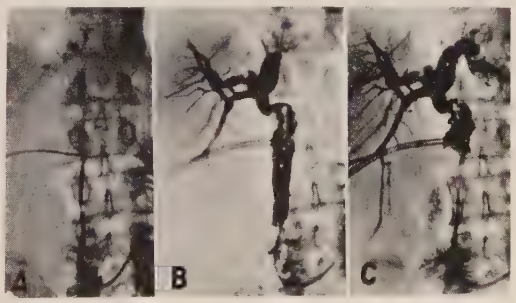


FIG. 2. Special T-tube after insertion into patient: (A) Distal tube entering duodenum, filled with contrast solution; (B) proximal tube is inj., contrast solution filling biliary tract and running around distal tube into duodenum; (C) balloon blown up, showing complete obstruction of common duct.

salts are reabsorbed, and recirculate in the physiological enterohepatic pattern. When total secreted bile is collected and not reinjected, measured secretion is found to average 400-500 ml. Under such abnormal conditions bile salts are not reabsorbed from the intestinal tract for reutilization. The liver has to manufacture bile salts each day anew. On the basis of physiological collection of bile as described here, the daily normal volume of biliary secretion in man varies between 1000 and 2000 ml. These figures are at variance with those suggested in text books of physiology, about 500 ml(2).

Total collection of human pancreatic juice is possible only if there is no functioning accessory duct of Santorini. That there is no such accessory duct can be ascertained by performing a pancreatogram either at operation, or in the post-operative period(3). Daily measured volumes of pancreatic juice

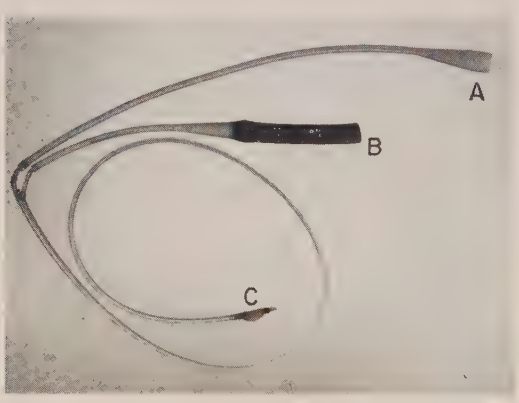


FIG. 3. Special tube for total pancreatic juice collection: Collection tube (A); sealed rubber tube end (B) of fine tube connected with tiny balloon (C), encircling end of collecting tube. Compression of rubber end causes distention of balloon, after it is inserted in terminal end of duct of Wirsung.

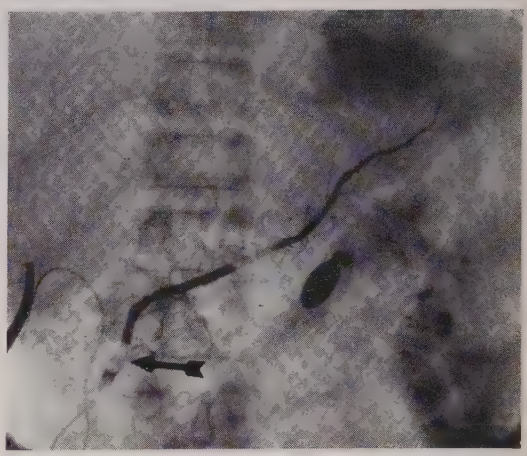


FIG. 4. Special tube for pancreatic juice collection after insertion into patient: Pancreatogram visualizes whole pancreatic duct, and outlines distended balloon (arrow).

secretion in man (1500-4000 ml) are at variance with the usual given figure of 700 ml mentioned in publications(4). Our figures were obtained from patients in whom the pancreas, from clinical evidence and by direct observation at operation, was normal.

Summary. Special tubes for measuring total output of bile and pancreatic juice in man under physiological conditions, are described. Bile secretion from normal livers varied between 1000 and 2000 ml; pancreatic juice from normal pancreas varied between 1500 and 4000 ml.

1. Doubilet, H., Mulholland, J. H., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 113.

2. Best, C. H., Taylcr, N. B., *Physiological Basis of Medical Practice,* Williams & Wilkins, 1953.

3. Doubilet, H., Poppel, M. H., Mulholland, J. H., *Radiology,* 1955, v64, 325.

4. Blumenthal, H. T., Probstein, J. G., *Pancreatitis,* Thomas, 1959.

Received October 20, 1960. P.S.E.B.M., 1961, v107.

Protein-Bound Hexosamine in Plasma and Erythrocytes of Normal and Diabetic Rats and Human Beings.* (26593)

Doris M. Hilker†, Kung-Ying Tang Kao, Elbert G. Bates and Thomas H. McGavack

Geriatrics Research Labs., Vet. Admin. Center, Martinsburg, W. Va., and Dept. of Medicine, George Washington Univ. Sch. of Medicine, Washington, D. C.

Protein-bound hexosamine, as a component of glycoprotein, has been found to vary greatly in a number of pathological conditions (1). Very little information, much of which is conflicting, is available concerning the relationship of these compounds to diabetes mellitus. Jacobs(2) reported that a mucoid fraction of blood plasma obtained by mild hydrolysis was increased in diabetic subjects and was decreased following administration of insulin in the same manner as glucose. Other investigators found the serum hexosamine content within normal range in diabetes uncomplicated by vascular degeneration(3, 4). In the diabetic rat, Spiro(5) reported no impairment in rate of synthesis of glucosamine in the liver, while in the skin, Schiller and Dorfman(6) observed a reduction in incorporation of C^{14}-labeled glucose into hyaluronic acid to one-third of that seen in the normal animal. There have been no reports on the hexosamine content of erythrocytes.

A preliminary study of protein-bound hexosamine concentrations in plasma and erythrocytes of normal and diabetic rats and human beings is reported here. The terminology used in this paper is according to Winzler(1).

Methods. Animals used in these experiments were male albino rats of the Wistar strain weighing 300-500 g. Alloxan diabetes was produced by intramuscular injection of 0.06 ml of a 10% solution of alloxan monohydrate in H_2O after a 48 hour fast. The animals were fed Purina rat chow *ad libitum* up to time of experiment.

Blood was collected from the tail into 1 ml teflon beakers, each of which contained 1 drop of dried Sequester-Sol as an anticoagulant. One ml of blood was transferred to a Wintrobe hematocrit tube by means of a Wintrobe pipette. Extreme care was taken to avoid hemolysis. The tubes were centrifuged at 2000 rpm for 30 min, after which the plasma was removed with a Wintrobe pipette and transferred to test tubes. The white cell layer was removed and discarded. The erythrocytes were washed 3 times with physiological saline, then diluted with saline to make a 1:1 suspension. The glycoprotein was precipitated by addition of 5 ml of 95% ethanol to 0.1 ml of the plasma samples, etc. and to 0.4 ml of the rbc suspensions in 12 ml centrifuge tubes. The tubes were centrifuged and the precipitate washed twice with 95% ethanol. Two ml of 3 N HCl were added to the precipitated proteins, the tubes fitted with air condensers and the contents hydrolyzed in a boiling water bath for 7 hours. One ml aliquots of the acid hydrolysates were neutralized with 4 N Na OH and salt concentration adjusted in the blank and standard tubes. Hexosamine determinations were made by the method of Elson-Morgan(7) as modified by Winzler(1). Optical density was determined at 530 mμ in a Coleman Jr. Spectrophotometer.

In some of the earlier experiments the hydrolysates were put through Dowex 50W ion exchange columns as described by Boas(8). It was found, however, that the hexosamine content of the plasma and red cells were essentially the same in the eluate from the ion exchange column as in the untreated hydrolysate (Table I). From these data it would appear either that little or no impurity was being measured when the Dowex column was not used, or that the Dowex column failed to remove any impurity which might have been present. In any event, the differences be-

* This investigation was aided by funds from Clinical Studies Div., Research Service, Veterans Admin., Washington, D. C., through a VA Cooperative Diabetes Study.

† Present address: Dept. of Food and Nutrition, Coll. Agri., Univ. of Hawaii, Honolulu.

TABLE I. Recovery of Hexosamine after Passage through Dowex 50W Column.

Sample No.	% of recovery* 1	2	3	4	5	6	7	Mean	S.D.
Glucosamine standard	101	97	91	102	100	97	99	98.1	3.7
Plasma hexosamine	101	99	102	99	101			100	1.4
Cell "	100	102	97	99				100	2.1
Plasma " + 125 μg glucosamine	94	94	99	106	98	99		99	4.9
Cell " + " " "	88	89	102	99	101	94		96	5.9

* Amount of material found in the aliquot analyzed without passage through the Dowex 50W column is in each instance arbitrarily assigned a value of 100%. Each percentage is derived by comparing the value obtained after chromatography with that found in the corresponding nonchromatographed specimen.

tween samples run through the column and those not so treated were not significant at the 5% level of confidence (Table I). Therefore, the use of Dowex 50W for determination of tissue hexosamine was abandoned. Our actions in this regard are in agreement with those of Rosenlund(9). No attempt was made to identify the particular hexosamine or group of hexosamines normally present in red cells.

Fasting human blood was collected from normal laboratory personnel and from known diabetic patients who were well controlled with diet and insulin or oral hypoglycemic agents.

Results. In Table II are shown the hexosamine content of plasma and erythrocytes and corresponding blood glucose values in normal and diabetic rats and human beings. The normal human plasma level was similar to that reported by Winzler(1). There was no significant difference between normal values and those of diabetic patients in either plasma or erythrocytes. Human hexosamine levels in both cells and plasma were lower than those in rats. The normal rats had significantly higher levels of hexosamine in both plasma and cells than the diabetic rats, which agrees with the work of Nichols *et al.*(10).

Discussion. In this study, the decreased hexosamine levels observed in cells and plasma of the diabetic rat differ from our own findings and those of others relating to diabetic patients, in whom either normal or increased serum mucoprotein concentrations were noted(3,4). However, a few cases of decreased serum mucoprotein in diabetic patients have been reported(12), although it was not stated whether or not other pathological conditions were present. In our hu-

TABLE II. Protein-Bound Hexosamine Levels in Plasma and Erythrocytes.

Group	Glucose, mg/100 ml	Hexosamine, mg/100 ml Plasma	Packed erythrocytes
		Rat	
Normal	127	154	53.0
	107	172	46.5
	136	156	41.5
	110	154	56.0
	114	160	52.5
		159.2 ± 7.6*	49.9 ± 5.8
Diabetic	424	137	36.5
	386	138	32.5
	304	145	50.0
	356	134	46.0
	270	142	35.7
	894	114	31.1
	258	152	41.8
		137.4 ± 11.9	39.1 ± 7.1
		Human	
Normal	95	92.5	22.6
	76	76.0	17.8
	81	81.7	21.6
	64	68.0	18.4
	89	88.5	18.2
	88	128.8	22.3
	76	114.6	19.3
	66	115.3	18.4
		95.7 ± 21.6	19.8 ± 2.0
Diabetic	126	78.9	20.4
	80	109.6	27.3
	64	93.6	20.1
	79	112.0	19.4
	61	102.2	22.6
	50	61.4	17.3
	108	114.0	19.3
	84	67.8	28.6
	38	69.0	27.3
		89.8 ± 20.9	22.5 ± 4.2

* Mean ± S.D.

The 5 normal rat plasma levels compared to 7 diabetic rat samples by the Fisher "t" test(11) gave $P < 0.01$; a comparison of the cells gave $P < 0.02$.

man subjects, the diabetes was well controlled at the time of observation.

Reductions in serum mucoprotein have been most frequently associated with impaired liver or endocrine function(13,14); our finding is a decrease in the protein bound hexosamine. Since the liver has been implicated as the primary site of glucosamine formation(15), it may be possible that these alloxanized rats have impaired liver function, resulting in a decreased synthesis of these compounds. The decreases in serum proteins, particularly a_1 globulin and albumin fractions in diabetic rats observed by Nichols *et al.*(10) are indicative of altered hepatic activity.

It has been reported that the liver of the diabetic animal as the primary site of glucosamine formation can synthesize hexosamines at a rate similar to the normal animal (5). If this were the case, the lower levels in the diabetic rat may be a reflection either of decreased synthesis or release of these compounds by secondary sites, such as connective tissue.

Summary. In both cells and plasma, alloxanized rats had significantly lower concentrations of protein-bound hexosamine than normal animals. In diabetic patients under insulin control, values were normal. Concentration of protein-bound hexosamine was twice as high in plasma and erythrocytes of normal rats as in normal human beings.

The authors gratefully acknowledge suggestions and advice of Dr. C. H. Treadwell.

1. Winzler, R. J., *Methods of Biochemical Analysis,* Vol. 2, Interscience Publishers, Inc., New York, N. Y., 1955, p279.

2. Jacobs, H. R., *J. Lab. Clin. Med.,* 1949, v34, 116.

3. Berkman, J., Rifkin, H., Ross, G., *J. Clin. Invest.,* 1953, v32, 415.

4. Shetlar, M. R., Foster, J. V., Kelly, K. H., Shetlar, C. L., Bryan, R. S., Everett, M. R., *Cancer Res.,* 1949, v9, 515.

5. Spiro, R. G., *Ann. N. Y. Acad. Sci.,* 1959, v82, 366.

6. Schiller, S., Dorfman, A., *J. Biol. Chem.,* 1957, v227, 625.

7. Elson, L. A., Morgan, W. T. J., *Biochem. J.,* 1933, v27, 1824.

8. Boas, N. F., *J. Biol. Chem.,* 1953, v204, 553.

9. Rosenlund, B., *Scand. J. Clin. and Lab. Invest.,* 1956, v8, 343.

10. Nichols N., Moinat, P., Tuller, E. F., *Diabetes,* 1956, v5, 468.

11. Mills, F. C., *Statistical Methods,* New York, N. Y., 1938.

12. Greenspan, E. M., *Arch. Int. Med.,* 1954, v93, 863.

13. Greenspan, E. M., Tepper, B., Terry, L. L., Schoenbach, E. B., *J. Lab. Clin. Med.,* 1952, v39, 44.

14. Greenspan, E. M., Lehman, I., Graff, M. M., Schoenbach, E., *Cancer,* 1951, v4, 972.

15. Spiro, R. G., *J. Biol. Chem.,* 1959, v234, 742.

Received November 14, 1960. P.S.E.B.M. 1961, v107.

Different Hemostatic Responses of Dogs to Heparin Injection.* (26594)

W. O. CRUZ, MECIA M. OLIVEIRA AND C. P. DIETRICH

Laboratory of Hematology, Instituto Oswaldo Cruz, Rio de Janeiro, Brasil

It is well known that the only effect which heparin has on hemostasis when it is injected intravenously in humans as in thrombosis is a drastic one on coagulation. The occurrence of rare accidents in heparinized patients has been interpreted as an individual susceptibility to hemorrhage while the blood remains incoagulable.

In our extensive observations on skin hemostasis in dogs, before and after injection of variable amounts of heparin, marked differences were noted not only with varying amounts of heparin, but also with a standard dose in different dogs. This difference in group response in dogs is described herein.

Methods. Unless otherwise stated the he-

* This work was aided by the Conselho Nacional de Pesquisas and by a grant from Dr. Guilherme Guinle.

TABLE I. Group Incidence of Skin Hemostasis Variation in Dogs after Intravenous Injection of Heparin (300 I.U./kg).

Heparin dog group	Bleeding time (min.)	Sex ♂ No.	♂ %	♀ No.	♀ %	No. not observed	Total No.	Total %
Resistant	5-12	254	63	194	59	57	505	63
Intermediate	13-29	75	18	67	20	4	146	18
Labile	30 or more	79	19	68	21	2	149	19
Total		408	—	329	—	63	800	—

parin employed in this investigation was Liquemin, Roche (5,000 I.U. per ml), diluted with saline and injected slowly intravenously. Other brands were Evans, Connaught, and Lutetia. Both anesthetized (sodium pentobarbital) and unanesthetized dogs were studied. Skin hemostasis was observed by the determination of bleeding time from safety razor blade cuts made, usually in the inner portion of the thigh, 10 minutes after the heparin injection, according to the technic previously described(8,9). Coagulation time was determined by the method of Lee and White(18). Heparin units refer to international units in which 100 I.U. are approximately equivalent to 1 mg of the barium salt of heparin.

The bleeding time of normal, untreated dogs varies from 5 to 11 minutes, with values of greater than 12 minutes very rare. Thus in this study the dogs, after a standard dose of 300 I.U. of heparin per kg, were divided into 3 groups: I, heparin-resistant dogs with bleeding times of less than 13 mins; II, heparin-intermediate dogs with bleeding times of greater than 13 minutes, but less than 30 minutes; and III, heparin-labile dogs with beeding times of more than 30 minutes.

Results. Incidence of a heparin effect. Table I shows the incidence of skin hemostasis disruption after intravenous administration of 300 I.U. of heparin per kg. The incidence is the same in both sexes, and if we consider all abnormal bleeding times, heparin administration at this dosage level interfered with the mechanism for skin hemostasis in slightly more than one-third of all dogs (37%).

Effect of anesthesia and surgical procedure. Bleeding and coagulation times in a heparin-labile dog which had received 580 I.U. of heparin per kg increased to more than 30 minutes. Anesthesia induced by sodium pentobarbital one hour after the heparin injection and continued for 3 hours did not alter either bleeding or coagulation time. After natural elimination of heparin (1.5 hr) and when both times had returned to normal levels, thoracotomy was performed and heparin once more injected (425 I.U. per kg). Bleeding and coagulation times rose to more than 30 minutes. One and one-half hours later both times again returned to normal. In 8 heparin-intermediate dogs the post-heparin bleeding times were 16, 20, 14, 16, 16, 21, 19, and 18 minutes before anesthesia. After anesthesia these values were 14, 40, 20, 16, 19, 28, 18, and 16 minutes, respectively.

Effect of different brands of heparin. Different brands of heparin were injected successively into a heparin-labile dog after bleeding and coagulation times had returned to normal following the preceding injection. Liquemin, Roche (B-508011), 425 I.U./kg; Liquemin, Roche (R-505027), 580 I.U./kg; heparin Evans (A-90590), 425 I.U./kg; and Lutetia, 300 I.U./kg in this order, resulted in similar bleeding and coagulation times of greater than 30 minutes.

A heparin-resistant dog received heparin Connaught (Lot 11081), 660 I.U./kg, and Liquemin, Roche (R-505027), 660 I.U./kg. With both Connaught and Liquemin heparin the dog had a normal bleeding time (4-8 minutes), but the blood remained incoagulable for more than 24 hours.

Fat feeding and heparin-lability. In one heparin-resistant and one heparin-labile dog, both of whom received 30 ml of olive oil by stomach tube and heparin intravenously at the peak of lipemia (4 hours after intubation), the coagulation time was greater than

TABLE II. Effect of Intravenous Heparin on Bleeding Time Increase in Heparin-Resistant Dogs.

Dog	Heparin (I.U./kg)	Bleeding time (min.)	Dog	Heparin (I.U./kg)	Bleeding time (min.)
1	300	6	6	300	12
	600	6		600	12
	1200	+30		1200	+30
2	300	10	7	300	9
	900	12		600	9
	1500	16		1200	15
	2100	25		1500	+30
3	300	23	8	300	4
	600	45		600	10
				900	11
4	300	9		1200	+30
	500	9			
	1000	7	9	300	5
	1300	8		600	8
	1700	10		900	13
	2000	23		1200	+30
	2300	+30	10	300	6
				600	+30
5	300	8			
	600	+30	11	300	8
				600	+30

12 hours. However, in the heparin-resistant dog the bleeding time was only 8-9 minutes, while in the heparin-labile dog it was greater than 30 minutes.

Heparin-lability and heparin dosage. Bleeding time increased with increasing dosage in heparin-resistant dogs (Table II). In Table III it is evident that the minimum dosage required to reveal heparin-lability varies considerably from one dog to another.

Persistence of heparin-response in same dog. In 2 dogs heparin-resistance persisted for at least 34 days following the standard dose of 300 I.U./kg. Similarly, heparin-lability persisted for at least 15 days.

TABLE III. Heparin-Lability in Different Dogs as Observed with Variable Amounts of Intravenous Heparin.

Dog	Heparin (I.U./kg)	Bleeding time (min.)	Coagulation time
A	300	+30	+12 hr
	100	11	+12 "
B	300	+30	+12 "
	100	9	+12 "
C	100	+30	22 min.
	50	18	20 "
D	110	+30	+30 "
	60	25	+30 "
	40	20	8 "
	20	10	5 "
E	300	+30	—
	50	19	—
	30	11	—
	0	7	—

Correlation between coagulation and bleeding times. As a crude test of rate of heparin elimination (detoxication), both coagulation and bleeding times were measured in some cases simultaneously. Table IV shows that degree of blood coagulability is independent of either heparin-resistance or -lability.

TABLE IV. Lack of Correlation between Coagulation and Bleeding Time in Resistant and Labile Dogs.

	Dog	Time after heparin inj.* (min.)	Coagulation time (hr)	Bleeding time (min.)
Resistant	1	25	12	8
	2	25	12	10
	3	25	12	8
	4	25	1	7
	5	30	12	9
	6	25	12	6
	7	25	12	6
	8	30	+½	6
	9	30	+½	7
Intermediate	10	45	12	23
	11	40	12	20
Labile	12	50	12	+30
	13	50	+½	+30
	14	45	+½	+30
	15	40	+½	+30

* Heparin in standard dose: 300 I.U./kg.

Discussion. The literature contains reports on the response of bleeding time in man and animals after heparin administration. According to one investigator the bleeding time remains normal in man after therapeutic doses of heparin(19), but another report describes a very small increase of bleeding time in man and rabbits following heparin administration(23). Of 10 rats injected with 500 I.U./kg, hemostasis did not occur in 3 animals. Even with a larger dose of heparin (2250 I.U./kg) hemostasis did not occur in 2 of 4 rats(24). No increase in bleeding time was observed in 5 rabbits who received 880 I.U./kg, in spite of an increase in coagulation time to 30 or 120 minutes(5).

The results described here show that in dogs heparin has, besides its effect on coagulation, the ability to interfere with control of skin hemorrhages, as assayed by bleeding

time determinations. Different commercial brands of heparin are equally active in this regard. We did not observe this in previous work when we reported that either heparin or citrate could be used equally well as an anticoagulant(8). Our previous failure to recognize this phenomenon may have been due to the fact that in this current study we found 63% of 800 dogs to be heparin-resistant, so that only the relatively few earlier observations could well have fallen in this group.

That heparin can induce platelet agglutination *in vitro*(6,7) or *in vivo* with thrombus formation(1-3,22) has been reported. Those workers state that thrombus formation depends upon the strength of the causative agent. Glass tubes, considered as strong agents, used in shunts induce thrombi, and thus keep bleeding time within normal limits. On the other hand, razor blade or needle injuries of the skin would not be strong enough to induce thrombus formation. Thus, according to those authors, prolonged bleeding occurs. This interpretation based on a thrombus as a causative agent in bleeding control when blood was heparinized could not, however, be applied to the phenomenon described herein, since dogs subjected to the same stimulus showed different hemostatic reactions (normal as well as prolonged bleeding times). Other work emphasized, moreover, that agglutination of platelets *in vitro* is related not to the presence of heparin itself, but to contaminating substances from heparin sources in early commercial preparations, since recent heparin products cannot influence platelet agglutination(15). Furthermore, in connection with thrombus formation and bleeding control, experimental studies employing the hind leg preparation for hemostasis studies(10) showed that arterial blood, devoid of platelets by reason of repeated and accurate fractional centrifugation, still keeps its hemostatic properties, casting some doubt on the necessity for the actual presence of a thrombus to induce hemostasis.

The doubt about platelet plugs functioning mechanically in bleeding control is reinforced by studies on the activity of plasma fractions which can convert non-hemostatic venous blood into hemostatic blood(11) or can inactivate arterial blood(12), as well as by observations in other hemostatic fields(13), all of which point to a direct and paramount role of plasma constituents in hemostasis, independent of agglutinated platelets. All of these studies are suggestive of an active plasma substance, acting biochemically rather than by direct mechanical occlusion, in control of hemorrhages of skin, small vessels and capillaries.

There is considerable literature on the effect of heparin on plasma proteins. The electrophoretic pattern of plasma proteins changes with amount of added heparin(17). The effect is very marked at high (0.4%) heparin concentration(4) when a component (Component C) appears to migrate between pure heparin and albumin. Anticomplementary activity of heparin has been studied(14). Plasma constituents involved in the heparin effect are thought to be, in man at least, mainly *beta*-globulin and possibly also $alpha_2$-globulin, whereas interaction with *gamma*-globulin and albumin is very slight by comparison(17). The plasma lipoprotein interaction is particularly interesting. It is well known that the clarifying effect of heparin discovered by Hahn(16) is not induced through a direct action of heparin upon plasma lipids but is mediated by a globulin component *in vivo*. Also alteration in the flotation rates of the lipoproteins of serum following heparin administration is detectable by ultracentrifugation(20). The active heparin-containing compound present in tissue mast cell cytoplasm has been extracted and purified(21) showing that heparin is found in a loose linkage with a polypeptide and a lipid which contains cholesterol, lecithin and neutral fats. This lipoprotein part of the molecule is very similar or identical to the heparin co-factor of blood serum according to electrophoretic data and clotting assays.

Our results show that if heparin activity is mediated by formation of a complex "heparin-hemostatic plasma component," this plasma component (rendered inactive by the complex formation) should be present at different concentrations in different dogs be-

cause, to suppress skin hemostasis completely (*i.e.*, a bleeding time of more than 30 minutes), each dog requires a different amount of heparin. Moreover, at least 2 other aspects of this hypothetical component are that there is constant concentration of it in each individual dog, at least for a period of weeks; and that its activity after injection of heparin is not associated with sex of the animal, with anesthesia, with extensive surgical procedures, or with lipemia caused by ingestion of large amounts of olive oil.

With a standard dose of heparin (300 I.U./kg) the distribution of heparin-lability in the dogs is fairly constant, several groups giving results very near the average incidence of 37% with bleeding time greater than 13 minutes. This seems to indicate that distribution of the plasma component rendered inactive by heparin is fixed in the species as a whole, but varies from one dog to another.

As the lability in dogs is quite variable, it is possible that responsiveness to heparin may vary also from one patient to another. Thus, if our findings could be extended to other mammals and to man, they might explain the abnormal bleeding of unknown mechanism that occurs in a percentage of surgical and thrombotic patients under heparin treatment.

Summary. Observations on bleeding time of 800 dogs after administration of a standard dose of heparin (300 I.U./kg) showed different responses which were divided into 3 groups: 63% as heparin-resistant dogs with normal bleeding time up to 13 minutes; 17% as heparin-intermediate dogs with the bleeding time between 13 and 30 minutes; and 18% as heparin-labile dogs with bleeding times higher than 30 minutes. This response of skin hemostasis does not depend on sex, anesthesia, extensive surgical procedures, heparin brand, or lipemia after olive oil feeding. A heparin-resistant or heparin-labile dog remains such after weeks of observation. The extent of lability varies in individual dogs, some showing an increased bleeding time even with comparatively small doses of heparin. On the other hand, heparin-resistant dogs could be made labile with increased amounts of heparin. It appears that heparin interferes with a plasma component normally active in the hemostatic mechanism. These results emphasize the paramount importance of plasma proteins, rather than a platelet thrombus, as the primary factor in control of skin hemorrhage in the small vessels. The possibility of similar findings in humans is stressed and the possible relationship of heparin-lability and some hemorrhagic accidents in heparinized patients is pointed out.

---

1. Best, C. H., *Harvey Lectures,* 1940, 41.
2. Best, C. H., Cowan, Maclean, *J. Physiol.,* 1938, v92, 20.
3. Best, C. H., Solandt, D. Y., *Lancet,* 1940, 1042.
4. Chargaff, E., Ziff, M., Moore, D. H., *J. Biol. Chem.,* 1941, v139, 383.
5. Chen, I. I., Tsai, C., *J. Physiol.,* 1948, v107, 280.
6. Copley, A. L., Robb, T. P., *Am. J. Physiol.,* 1941, v133, 248.
7. Copley, A. L., *Fed. Proc.,* 1948, v7, 22.
8. Cruz, W. O., Oliveira, A. C., *J. Appl. Physiol.,* 1958, v13, 368.
9. Cruz, W. O., *The Coagulation of Blood, Methods of Study.* Edited by L. M. Tocantins, Grune & Stratton, 2nd Ed. to be published.
10. Cruz, W. O., Oliveira, A. C., *Brit. J. Haem.,* 1959, v5, 368.
11. Cruz, W. O., Magalhães, J. R., Oliveira, Mecia M., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 740.
12. Cruz, W. O., Magalhães, J. R., Meis, L., *Blood,* 1960, v16, 1433.
13. Cruz, W. O., Studies on Hemostasis *in vivo,* presented in Symposium on platelets. Ford Memorial Hosp. 1960, Detroit. Edited, 1961, by Little, Brown & Co., Boston, Mass.
14. Ecker, E. E., Pillemer, L., *J. Immunol.,* 1941, v40, 73.
15. Fidler, E., Jaques, L. B., *J. Lab. Clin. Med.,* 1948, v33, 1410.
16. Hahn, P. F., *Science,* 1943, v98, 19.
17. Hoch, H., Chanutin, A., *J. Biol. Chem.,* 1952, v197, 503.
18. Lee, R. I., White, P. D., *Amer. J. Med. Sci.,* 1913, v45, 495.
19. MacFarlane, R. G., *Quart. J. Med.,* 1941, v33, 1.
20. Nikkila, E., *Scand. J. Clin. and Lab. Inv.,* 1953, v5, suppl. 8.
21. Snellman, O., Sylvén, B., Julén, C., *Biochem. et Biophys. Acta,* 1951, v7, 98.

22. Solandt, D. Y., Best, C. H., *Lancet*, 1938, v2, 130.

23. Roskam, J., *Arch. Intern. Pharmacodyn. et Thérap.*, 1942, v68, 66.

24. Zucker, Marjorie B., *Am. J. Physiol.*, 1947, v148, 275.

Received January 3, 1961. P.S.E.B.M., 1961, v107.

Metabolism of Tyramine by Smooth Muscle Preparations *in vitro*.* (26595)

MARILYN W. MCCAMAN† (Introduced by David M. Gibson)

Department of Pharmacology, Washington University School of Medicine, St. Louis, Mo.

The metabolism of amines, particularly sympathomimetic amines, has been of considerable interest in recent years. In smooth muscle 2 pathways by which these amines are known to be inactivated are oxidative deamination and O-methylation(1,2). Evidence will be presented here for the presence of a new metabolic pathway in smooth muscle which converts p-hydroxy-phenylethyl amines to catecholic compounds. These results were obtained from a study of the metabolism of tyramine by rabbit aorta and stomach muscle.

Materials and methods. Fresh rabbit aorta (descending thoracic and abdominal) was rinsed with buffer, trimmed, and cut into longitudinal pieces. Strips of stomach muscle were prepared by making parallel incisions through the muscle layers only and gently teasing the muscle away from the submucosa. All procedures were carried out at 2-4°C. Incubation flasks (25 ml Erlenmeyer), containing 200 μM oxygenated Na phosphate buffer, pH 7.4, and 20 μM tyramine in a total volume of 2.0 ml, were mounted in a rack and oxygenated by bubbling 100% O_2 below the surface of the fluid for 5 minutes. Strips of tissue were added (100-200 mg wet weight), and the flasks securely stoppered and incubated at 38° with shaking. The incubation was terminated by addition of HCl (final concentration 0.2 N) after removing the tissue (usually after 240 min). Acidified samples were used directly for all chemical and chromatographic analyses. Flasks incubated without substrate or without tissue were included as controls in each experiment.

All spectrophotometric determinations were made with the Beckman DU spectrophotometer fitted with a diaphragm and microcells. For ultraviolet absorption spectra, solutions were read in 0.3 N HCl and 0.3 N NaOH at a dilution sufficient to obtain an optical density of 0.200-0.800.

Procedures used for determination of amine(3) and p-hydroxyphenol(4) groups have previously been described. In the latter method, tyramine (Mann), tyrosine (Fisher), N-methyltyramine (Smith, Kline and French), p-hydroxyphenylacetic acid (Aldrich) and p-hydroxyphenylethanol (synthesized according to Ferber)(5) gave approximately the same molar extinction coefficient(ϵ). p-Hydroxyphenylethanolamine (Winthrop-Stearn), p-hydroxymandelic acid‡ and p-hydroxybenzaldehyde (Eastman) gave about 20% of the color obtained with tyramine. Epinephrine, norepinephrine, cobefrine, 3-hydroxytyramine, and neosynephrine (all from Winthrop-Stearn) gave no significant color under these conditions.

For chromatographic analysis, aliquots of acidified samples (10-2000 μl) were put directly on Whatman #1 filter paper, 5-15 μl at a time. The following solvents were used: 1) n-butanol-glacial acetic acid-water, 4:1:1;

* This work was completed during the tenure of a fellowship of the Life Insurance Medical Research Fund. The data in this paper formed part of a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Washington University, 1957.

† Present address, Inst. of Psychiatric Research and Dept. of Neurology, Indiana Univ. Med. Center, Indianapolis.

‡ Kindly furnished by Dr. Sidney Udenfriend, Nat. Inst. Health, Bethesda, Md.

2) isopropanol-conc. NH_4OH-water, 8:1:1; and 3) chloroform-glacial acetic acid-water, 2:1:1, organic layer. The spray reagents(6) were: for phenols, diazotized sulfanilic acid (DSA); for amines, ninhydrin in butanol; for catechols, ethylene diamine; and for aldehydes, 2,4-dinitrophenylhydrazine (Di-NO_2-PH).

Properties of p-hydroxyphenylacetaldehyde (HPA-ald). To determine the chromatographic and ultraviolet absorption characteristics of HPA-ald, a series of experiments were carried out using a partially purified amine oxidase (AO) preparation from steer plasma. This enzyme, which catalyzes the oxidative deamination of numerous amines including tyramine, has been measured by the ultraviolet absorption (240-280 mμ) of the aldehydes formed from benzylamine, furfurylamine and homosulfanilamide(7). AO§ (2 mg in 0.5 ml H_2O) was added to 150 μM Na phosphate buffer, pH 7.2, 4 μM tyramine, and 1 unit purified catalase in a volume of 1.5 ml. After oxygenation and incubation for 240 min, approximately 40% of the tyramine had been metabolized. Values for the p-hydroxyphenol group did not change significantly during the incubation. Addition of Di-NO_2-PH (0.1% in 2 N HCl) produced a small amount of a yellow precipitate, and the clear red solution obtained after NaOH had an absorption peak at 480 mμ, which is typical of carbonyl compounds(8,9).

The ultraviolet absorption spectrum of the incubation mixture revealed one new peak at 330 mμ in addition to the tyramine peak at 295 mμ. Both the ultraviolet-absorbing material and the Di-NO_2-PH-reacting material could be extracted quantitatively into organic solvents from an acidic solution (<pH 7) but not from an alkaline solution (>pH 10). Evaporation of the solvent layer left a film of sweet smelling, yellowish oil. The increases in optical density of the incubation mixture at 330 mμ and the Di-NO_2-PH color at 480 mμ are given in Table I. The ratio of these absorptions remained constant during the incubation time. Chromatograms showed only one phenolic spot (R_f's of .96, .94 and .92 in solvents 1, 2 and 3, respectively) in addition to tyramine (R_f's of .37, .78 and .00, respectively). The former spot was also detected after spraying with Di-NO_2-PH.

TABLE I. Increase in Optical Densities during Incubation of Tyramine with AO.

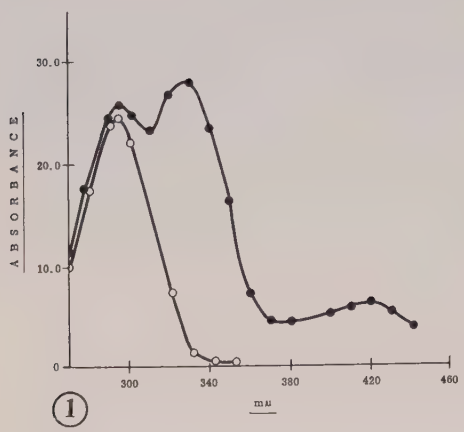
At each time indicated, 18 μl samples were diluted to 200 μl by addition of 0.3 N NaOH for the 330 mμ absorption and by addition of Di-NO_2-PH and 2 N NaOH for the Di-NO_2-PH color. The latter was read at 480 mμ. Each value represents the difference between the optical density of the incubated sample and the zero time sample.

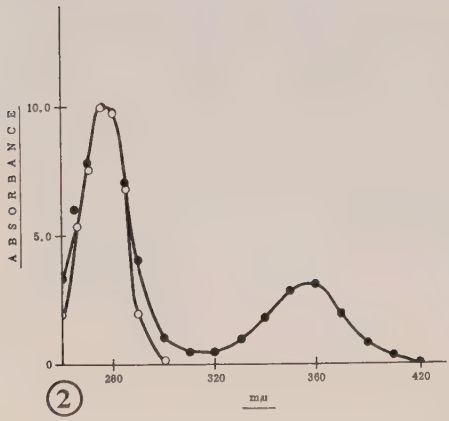
Absorption	Min. of incubation 60	180	240
A. 330 mμ	.191	.595	.815
B. Di-NO_2-PH	.067	.206	.276
A/B	2.85	2.88	2.92

The oxidation of this aldehyde to HPA-acid was demonstrated by reincubating a portion of the ether-soluble fraction of the AO incubation mixture with 100 mg rabbit aorta. This fraction was prepared by extracting the incubation mixture with ether, evaporating with a stream of N_2, and redissolving the residue in 2 ml phosphate buffer, pH 7.4. The optical density at 330 mμ decreased from an initial reading of 0.610 to 0.197 in 4 hours, while the hydrazone color decreased from a reading of 0.215 to 0.066 (330 mμ O.D./Di-NO_2-PH = 2.94). The total p-hydroxyphenol did not change. Whereas the chromatograms of the solution before incubating with aorta showed a single fast-moving phenolic spot in all 3 solvents (see above), after incubation the intensity of this spot decreased and a second phenol, identified as HPA-acid, was found (R_f's of .78, .44 and .82 in solvents 1, 2 and 3, respectively). These R_f's were identical to those found with the authentic acid run under the same conditions, and after addition of the known compound to the incubation mixture this area remained as a single spot in each solvent.

On the basis of this evidence it was concluded that the product of AO action on tyramine, HPA-ald, has a strong ultraviolet absorption peak at 330 mμ and gives R_f's of .96, .94 and .92 in solvents 1, 2 and 3, respectively.

§ AO (Stage 4, containing 5 manometric units/mg) was kindly furnished by Dr. Herbert Tabor, Nat. Inst. Health, Bethesda, Md.





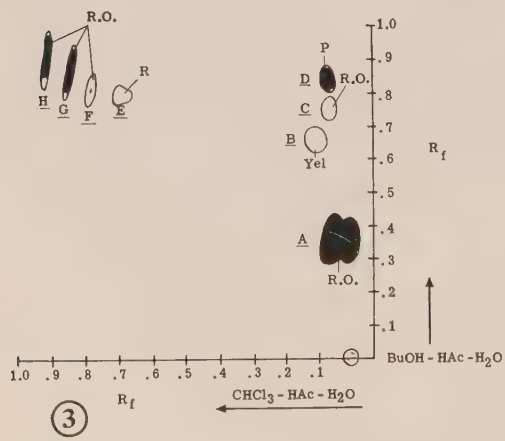


FIG. 1. Absorption spectrum of incubation mixture in alkali. Absorption spectrum of incubation mixture was obtained after diluting an aliquot with 0.3 N NaOH. Optical density in figure is calculated back to original concentration of incubation mixture. Tyramine, ○—○; incubation mixture, ●—●.

FIG. 2. Absorption spectrum of incubation mixture in acid after prior treatment with alkali. Absorption spectrum of incubation mixture was obtained by first diluting an aliquot in 0.3 N NaOH, then acidifying the solution (final concentration approximately 0.3 N HCl excess). Optical density as in Fig. 1. Tyramine, ○—○; re-acidified incubation mixture, ●—●.

FIG. 3. Two-dimensional chromatogram of a tyramine-aorta incubation mixture. Spray reagent, DSA and Na_2CO_3. Tyramine, A; HPA-acid, G; HPA-ald, H; tentatively identified as 3,4-dihydroxyphenylacetic acid, D; unknown, B, C, E, and F. Colors: R.O., red-orange; R, red; P, purple; Yel, yellow.

Results. In a series of 8 experiments with aortic strips, the average rate of tyramine disappearance by amine determination was 67.9 (range 55-80) μequivalents (μeq)/g wet weight aorta per 4 hr. The p-hydroxyphenol reaction disappeared at an average rate of 45.9 (range 31-75) μeq/g per 4 hr. The values obtained with stomach muscle were approximately the same as those with aorta. No disappearance of amine or p-hydroxyphenol reaction was observed in flasks containing boiled tissue (5 min at 100°). No evidence could be found for conjugation of the p-hydroxyphenol group, accumulation of drug or metabolites in muscle water, or metabolism of HPA-acid. Purified catalase (1 unit) added to incubation flasks did not alter the amine or p-hydroxyphenol disappearance. There was no apparent metabolism of tyramine in 100% N_2, and the rate in air for these reactions was 20-25% of the rate in oxygen.

A few preliminary experiments were done with homogenates of rabbit aorta. During the incubation, amine disappeared and aldehyde was formed (positive 2,4-dinitrophenylhydrazone test). This activity was found primarily in the residue obtained from centrifugation at 6000 × g for 30 min in the cold. No change in the p-hydroxyphenol reaction could be demonstrated in these homogenates.

During this investigation, it was observed that addition of NaOH to a colorless incubation sample produced a bright yellow solution. The ultraviolet spectrum of this alkaline sample revealed 2 absorption peaks in addition to that of tyramine.

The absorption spectrum of tyramine in 0.3 N HCl shows a single peak at 275 mμ ($\epsilon = 1200$); in 0.3 N NaOH the peak is found at 295 mμ ($\epsilon = 2500$). (Fig. 1). The

spectrum of closely related p-hydroxyphenyl compounds including tyrosine, N-methyltyramine, p-hydroxyphenylacetic acid, p-hydroxyphenylethanol, N-methyl-p-hydroxyphenylethanolamine, and p-hydroxyphenylethanolamine was almost identical to that of tyramine. Epinephrine, norepinephrine, 3-hydroxytyramine, epinine, cobefrine, and neosynephrine also gave a similar spectrum in acid solution; however, with the exception of neosynephrine, these latter compounds were unstable in alkali.

Fig. 1 shows the absorption spectrum of an incubated sample. In addition to the tyramine peak at 295 mμ, 2 new peaks were observed with maxima at 330 and 420 mμ. The increase in optical density at 330 mμ was proportional to the increase in Di-NO_2-PH color of the incubation mixture. The compound giving the peak at 330 mμ has been identified as HPA-ald (see *Methods*). The nature of the compound giving the 420 mμ peak is not known. Since alkaline solutions containing only HPA-ald do not show any absorption in this region, this peak may represent a previously unknown metabolic product of tyramine.

The absorption spectrum of an incubated sample in acid is identical with the tyramine curve except for some nonspecific absorption at the lower wavelengths. If a sample is reacidified after alkaline treatment, however, the original acid spectrum is not reproduced. In addition to the original absorption peak at 275 mμ (tyramine and HPA-ald), a new peak was observed at 360 mμ (Fig. 2). A second addition of NaOH reproduced the original alkaline spectrum (Fig. 1).

A typical chromatogram of an incubation mixture is reproduced in Fig. 3. The R_f region between .75 and .90 in solvent 1 was resolved into at least 6 phenolic compounds after chromatographing in solvent 3. Spot "A" represents unchanged tyramine. "G" is HPA-acid, identified by comparison with the known compound (R_f's = .78, .42 and .82 in solvents 1, 2 and 3, respectively; when added to the incubation mixture, the authentic acid and "G" remained as a single spot). "H" is probably HPA-ald (R_f's = .96, .93, .90, respectively, see *Methods*). The spot labelled "D" may be 3,4-dihydroxyphenylacetic acid, since the R_f's found in the 2 solvents agree with those reported in the literature (R_f of .85 in solvent 1 and .09 in solvent 3)(10,11), and the purple color is typical of catechols. The red-orange color of "C", "E", and "F" obtained with the DSA reagent has been seen with many p-hydroxyphenyl compounds, while the yellow color of "B" has been seen with many m-hydroxyphenyl compounds. The R_f's of these compounds do not correspond to any reported in the literature. In incubation mixtures containing boiled tissue, only unchanged tyramine could be detected.

Discussion. The disappearance of the p-hydroxyphenol reaction of tyramine reported here for rabbit aorta and stomach muscle has been previously observed in rabbit heart and intestine and in dog aorta(12,13). These authors suggested that this disappearance was due to a cleavage of the phenyl ring of tyramine. Such a reaction might produce compounds similar to the unsaturated aliphatic acids which are formed from other phenolic and catecholic compounds(14,15,16). However, preliminary studies indicated that when tyramine-8-C^{14} was incubated with rabbit aorta, the pattern of spots obtained from radioautographs of the chromatograms was not significantly different from that found on the DSA-sprayed chromatograms, which reveals only phenols.

Since all of the compounds tested which contain a p-hydroxyphenylethyl nucleus gave essentially the same amount of color in the procedure used (see *Methods*), the disappearance of p-hydroxyphenol groups probably results from formation of new compounds which produce less color in the analytical method. At least 5 new phenolic compounds have been found in the medium after incubation of tyramine with aorta. Since no metabolic products are found in presence of boiled tissue, these new compounds may be the result of new and uncharacterized enzyme systems in smooth muscle. One of these compounds has been tentatively identified as 3, 4-dihydroxyphenylacetic acid.

Pisano, Creveling and Udenfriend(17) have recently reported the β-hydroxylation of tyramine to norsynephrine in adrenal me-

dulla slices and other tissues. Although norsynephrine *per se* was not one of the unknown compounds found in the incubation mixture, perhaps Spot B, which gives color reactions similar to norsynephrine (*i.e.*, yellow with DSA), represents a metabolite of this compound. In the present study it was impossible to separate HPA-ald from p-hydroxyphenylethanol chromatographically so that the presence or absence of the alcohol could not be determined. Goldstein *et al.*(18) reported the appearance of 3-methoxy-4-hydroxyphenylethanol as an end product of dopamine metabolism.

The rate of disappearance of the p-hydroxyphenol reaction is only slightly less than that observed for amine disappearance (monoamine oxidase activity), which suggests that this pathway is important in the metabolism of tyramine by smooth muscle. It also supports the hypothesis that the pharmacological action of tyramine may result from both a direct effect on the receptor sites and an indirect effect by conversion to a more potent sympathomimetic amine, such as norsynephrine or dopamine(19).

Summary. During incubation of tyramine with rabbit aorta or stomach muscle, tyramine is metabolized as evidenced by a significant decrease in the amount of p-hydroxyphenyl and primary amine groups of tyramine and by concomitant changes in the ultraviolet absorption spectrum of the incubation mixture. Chromatographic analysis revealed the appearance of p-hydroxyphenylacetaldehyde and p-hydroxyphenylacetic acid as well as 5 unknown phenolic spots. One of these spots has been tentatively identified as 3,4-dihydroxyphenylacetic acid. The decrease of p-hydroxyphenyl groups of tyramine is probably accounted for by its conversion to dihydroxy or β-hydroxy compounds which given less color in the analytical procedure used.

The author wishes to thank Dr. Robert F. Furchgott for his interest and encouragement during this investigation.

1. Thompson, R. H. S., Tickner, A., *J. Physiol.*, 1951, v115, 34.

2. Axelrod, J., Tomchick, R., *J. Biol. Chem.*, 1958, v233, 702.

3. Robins, E., Lowry, O. H., Eydt, K. M., McCaman, R. E., *ibid.*, 1956, v220, 661.

4. Axelrod, J., *J. Pharmacol. Exp. Therap.*, 1954, v110, 315.

5. Ferber, A., *Ber. Dtsch. Chem. Ges.*, 1929, v62, 190.

6. Block, R. J., Durrum, E. L., Zweig, G., *Manual of Paper Chromatography*, Acad. Press, New York, 1955.

7. Tabor, C., Rosenthal, L., Tabor, H., *J. Biol. Chem.*, 1954, v208, 645.

8. Weissbach, H., Redfield, B., Udenfriend, S., *ibid.*, 1957, v203, 953.

9. Friedemann, T. E., Haugen, G., *ibid.*, 1943, v147, 415.

10. Armstrong, M. C., Shaw, K. N. F., Wall, P. E., *ibid.*, 1956, v218, 293.

11. Dagleisch, C. E., *Arch. Bioch. & Biophys.*, 1955, v58, 214.

12. Bernheim, F., Bernheim, M. L. C., *J. Biol. Chem.*, 1944, v153, 369.

13. ———, *ibid.*, 1945, v158, 425.

14. Knox, W. E., Edwards, S. W., *ibid.*, 1955, v216, 489.

15. Stannier, J., *J. Bact.*, 1950, v59, 527.

16. Wiss, O., *Z. Physiol. Chem.*, 1956, v304, 221.

17. Pisano, J. J., Creveling, C. R., Udenfriend, S., *Biochim. Biophys. Acta*, 1960, v43, 566.

18. Goldstein, M., Friedhoff, A. J., Pomerantz, S., Simons, C., *ibid.*, 1960, v39, 189.

19. Furchgott, R. F., *Pharm. Rev.*, 1955, v7, 183.

Received January 19, 1961. P.S.E.B.M., 1961, v107.

Alimentary Absorption of Reducing Sugars by Embryos and Young Chicks.* (26596)

PHYLLIS HOLT BOGNER† (Introduced by P. L. McLain)
Poultry Dept., University of Maryland, College Park

The aim of this research was two-fold. It sought to determine the relative absorption rates from the chick alimentary tract of a variety of sugars, including 6 pentoses, 5 hexoses, a heptose and a disaccharide. On the basis of this information several sugars differing in both molecular size and absorption characteristics were selected for an exploratory study of intestinal absorptive capacity of embryos and newly-hatched chicks. In mammals, studies of intestinal absorptive function during the transition from pre- to postnatal life are encumbered by the inaccessibility of the fetus. This difficulty was obviated by choosing the chick as the experimental animal.

Materials and methods. The embryos and chicks used throughout this investigation were of a cross of White Leghorn males to Flightless females and were unsexed. Alimentary absorption rates of 13 reducing sugars in aqueous solution (18% w/v) were determined by the Cori method(1) essentially as outlined for the chick by Golden and Long (2).

The following procedure was devised for experiments involving embryos. Eggs incubated for 17 days were opened at the blunt end. Membranes above the embryo were peeled back and the blood vessels carefully parted to allow raising of the embryo's head. After delivery of the sugar solution *via* a catheter into the crop, the head was stabilized outside the egg by means of a collar of aluminum foil (which also served to minimize evaporation of the egg's contents). The embryo was then returned to the incubator for the absorption interval. Thereafter the embryo was lifted from the egg and extra-embryonic vessels were severed. The body cavity was immediately opened, the heart removed, and adhering blood washed away. Sugar was recovered from the alimentary tract first by perfusion and then by washing after slitting it open *in situ*.

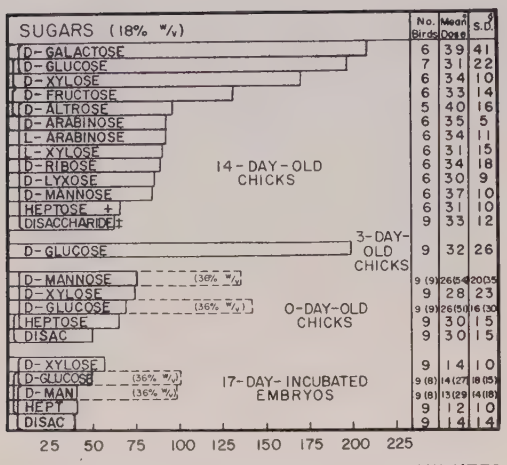
The volumes of sugar introduced into the crops of the 14-, 3- and 0-day-old chicks and embryos were 2.0, 0.6 and 0.15 ml, respectively. Chicks were not permitted access to food for 24 hours prior to experiments. The quantity of sugar absorbed was determined by computing the difference between the amount fed and that recovered from the entire alimentary tract after a 30 min absorption period. Absorption rates were expressed as an absorption coefficient, *viz.*, mg of sugar absorbed/100 g body weight/30 min. Body weights were exclusive of yolk-sac weights. Mean absorption rates of sugars were based on no less than 2 separate experiments on different lots of chicks. Quantitative determinations of reducing sugars were carried out according to the method outlined by Somogyi(3).

Results. Relative absorption rates of sugars fed to the experimental birds are graphically represented in Fig. 1. To the right of each bar is tabulated the number of chicks, mean dose of sugar administered and standard deviation of the mean absorption rate for that group. Sugars within a single bracket were not absorbed at significantly different rates as determined by the Multiple Range Test of Duncan(4).

Results obtained with 14-day-old chicks indicated that D-galactose, D-glucose, D-xylose and D-fructose were absorbed at significantly faster rates than the 9 remaining sugars tested, with the sole exception that D-fructose and D-altrose were not significantly different. Three-day-old chicks also absorbed

* Material taken from a thesis submitted to the Faculty of the Graduate School of the University of Maryland in partial fulfillment of the requirements for the degree of Doctor of Philosophy, presented in part before Am. Physiol. Soc., Philadelphia, Pa., Apr., 1958.

† Present address: School of Medicine, Univ. of Pittsburgh, Dept. of Physiology and Pharmacology, Pittsburgh, Pa.



* mg/10 g body wt.
† D-glycero-D-galacto-heptose.
‡ Cellobiose.
§ Stand. dev. of mean absorption rate.

FIG. 1. Mean alimentary absorption rates of sugars fed to chicks. Differences between sugars within a single bracket were not significant at $P = 0.05$.

glucose rapidly at a rate similar to that of the older birds. Henceforth D-galactose, D-glucose, D-xylose, and D-fructose will be designated as rapidly absorbed and all the other sugars as slowly absorbed.

Based on the above findings, 2 rapidly absorbed and 3 slowly absorbed sugars of differing molecular size were selected for the experiments with embryos and 0-day-old chicks. These results (Fig. 1) showed that 1) since D-glucose and D-xylose were absorbed at the same slow rates as the other sugars of similar size, at these early ages the sugars could not be segregated into rapidly and slowly absorbed groups; 2) there was a tendency for the largest sugars (the heptose and disaccharide) to be the most slowly absorbed, suggesting an inverse relationship between absorption rate and molecular size; 3) when concentration (hence the dose) of D-glucose and D-mannose was doubled, absorption rate was proportionately elevated, so that even at these higher dose levels there was no apparent difference between the "rapidly" absorbed and "slowly" absorbed sugars; and 4) adjustment of the glucose absorption coefficients of the embryos and 0-day-old chicks to a dose of 31 mg/10 g body weight, for comparison with the 14-day-old chicks fed this sugar, gave the values 107 and 82, respectively. Clearly these adjusted absorption coefficients are closer to those of sugars slowly absorbed by the older birds than to that recorded for glucose.

Discussion. The results of a survey comparing alimentary absorption rates of 13 sugars in 14-day-old chicks showed that the rates for these sugars decreased (without regard for statistical significance) in this order: D-galactose, D-glucose, D-xylose, D-fructose, D-altrose, D- and L-arabinose, L-xylose, D-ribose, D-lyxose, D-mannose, heptose, and disaccharide. Of the 4 most rapidly absorbed, the rate for D-galactose did not differ significantly from that for D-glucose, but it was significantly greater than that either for D-xylose or D-fructose. D-glucose differed significantly only from D-fructose. Of the 9 most slowly absorbed, the rate for only D-altrose was not significantly less than for that of D-fructose, the slowest of those rapidly absorbed. The observed differences in absorption rates for compounds of similar size suggest that the rapidly absorbed sugars—D-galactose, D-glucose, D-xylose and D-fructose—are selectively absorbed by this species. The absorptive capacity of the chick, with regard to sugars, therefore resembles closely that of the pigeon(5) and the mammal(6).

Studies with the 0-day-old chicks and embryos indicated that selective absorption does not occur at these ages, at least not to an extent measurable by the technique employed. The uniformly slow absorption velocities of all sugars tested, including D-glucose and D-xylose, suggest a passive mode of absorption. If, indeed, the laws of simple diffusion prevail at these ages, then the negligible differences between the absorption rates of the sugars are only an expected consequence of the equally small differences in their molecular size. Even so, there was a tendency for the largest molecules to be the most slowly absorbed.

In contrast, chicks only 3 days of age absorbed glucose as rapidly as the 14-day-old chicks, or at a rate more than twice that

calculated for 0-day-old chicks fed a comparable dose of the sugar.

These data suggest that the special mechanisms invoked to explain the selective absorption of sugars such as D-glucose are probably not fully functional until some time between 1 and 3 days after hatching.

Summary. A comparison of the alimentary absorption rates of 13 sugars in 14-day-old chicks indicated that D-galactose, D-glucose, D-xylose and D-fructose are selectively absorbed by this species. Furthermore, the ability to selectively absorb sugars seems to be fully developed in chicks just 3 days of age. In contrast to these older birds, embryos and 0-day-old chicks absorbed D-glucose and D-xylose at rates suggestive of a passive mode of absorption. These data have been interpreted as meaning that the selective absorptive capacity of the chick gut is not maximally developed until 1 to 3 days after hatching.

The author is indebted to Dr. Frank H. Wilcox for his guidance during this investigation.

1. Cori, C. F., *J. Biol. Chem.,* 1925, v66, 691.
2. Golden, W. R. C., Long, C. N. H., *Am. J. Physiol.,* 1942, v136, 244.
3. Somogyi, M., *J. Biol. Chem.,* 1952, v195, 19.
4. Duncan, D. B., *Biometrics,* 1955, v11, 1.
5. Westenbrink, H. G. K., *Arch. néerl. Physiol.,* 1936, v21, 433.
6. Crane, R. K., *Physiol. Rev.,* 1960, v40, 789.

Received January 30, 1961. P.S.E.B.M., 1961, v107.

Development of Intestinal Selective Absorption of Glucose in Newly-Hatched Chicks.*† (26597)

PHYLLIS HOLT BOGNER AND I. ANN HAINES (Introduced by P. L. McLain)
Department of Physiology and Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

In an exploratory study of alimentary absorptive function in young chicks, the absorption velocity of glucose was found to increase by several fold between 0 and 3 days of age (1). The aim of the experiments described below was to characterize more fully this apparent change in absorptive capacity which occurs just after hatching. Accordingly, the absorption rates of glucose and sorbose, each alone and each in the presence of phlorhizin, were compared in chicks between 0 and 10 days of age. Sorbose, a passively absorbed sugar(2), was selected to obtain an estimate of the amount of hexose which leaves the intestine by simple diffusion. Any glucose absorbed in excess of this amount would presumably reflect the activity of selective absorption mechanisms. Phlorhizin was chosen because of its relatively specific ability to inhibit glucose absorption(3). It was of interest to see if its potency, in this respect, might vary with the changing functional capacity of the absorbing cells.

Materials and methods. Chicks, used without regard to sex, were obtained from a local hatchery and were of a Mount Hope-Babcock strain cross. Alimentary absorption rates were determined by the Cori method(4) essentially as adapted for the chick by Golden and Long(5). In these experiments the sugar absorbed was estimated by the difference between the quantity of sugar initially introduced into the crop and that recovered from the entire alimentary tract after a 30 minute absorption interval. In the recovery procedure a clamp was placed at the uppermost part of the duodenum so that the sugar retained above and below this point could be measured separately.

Sugars were administered as 10% solutions in distilled water. When phlorhizin was used, it was added to the sugar solution in final

* Aided by grants from U.S.P.H.S. and the Smith Kline and French Fn.

† Presented in part before Am. Physiol. Soc., Atlantic City, N. J., Apr., 1959.

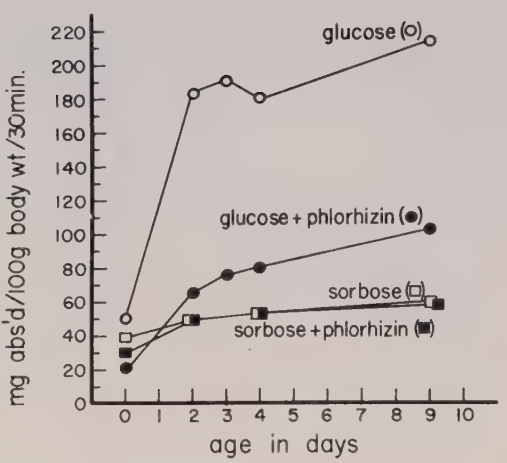


FIG. 1. Alimentary absorption rates of certain sugars in young chicks.

concentration of 0.002 M. The dose of sugar was adjusted as nearly as possible to 28 mg/10 g body weight. Absorption rates were expressed both as an absorption coefficient, *viz.*, mg sugar absorbed/100 g body weight/30 minutes, and as a percentage of total amount of sugar administered. Mean absorption rates for each experimental group were based on 10 or more individual determinations carried out on at least 3 separate lots of chicks. Sugar analyses were carried out according to the Somogyi-Nelson method(6).

Results. Experimental findings, summarized in Fig. 1 and Table I, indicate that:

1. Sorbose was absorbed by chicks in all age groups at a uniformly slow rate which was uninfluenced by the presence of phlorhizin. These results are in keeping with the view that this inert sugar is passively absorbed from the intestine(2).

2. Chicks 2 days of age and older absorbed glucose about 4 times as rapidly as 0-day-old chicks. Since the elevated absorption rates were not associated with a comparable increase in sugar delivered into the intestines proper (Table I), they presumably resulted from a greater absorptive capacity on the part of the intestinal epithelium.

3. Zero-day-old chicks absorbed glucose as slowly as sorbose. Calculations indicate that only about 1/5 of the glucose presented to the intestinal mucosa during the absorption interval was actually absorbed. It is therefore unlikely that its absorption was limited by a lack of substrate in contact with the absorbing surface. That glucose, as well as sorbose, is absorbed principally by a passive process at this early age would thus seem to be suggested.

4. However, administration of phlorhizin led to a 52-65% reduction in glucose absorption rate at all ages. As a consequence, in 0-day-old chicks, this served to depress the glucose absorption to a rate significantly below ($P = 0.05$) that of sorbose whether this latter sugar was fed alone or with phlorhizin. Hence it would appear that, while the absorption rates of glucose and sorbose are similar on the day of hatching, there may well be a difference between the two sugars in the precise mechanism of absorption.

Discussion. The absorption velocity of glucose from the chick intestine increased sharply after hatching, achieving a maximal level within just 48 hours. This increase served to raise the ratio of absorbed glucose to sorbose—a sugar presumed to be passively absorbed—from 1:1 to 4:1. It was further shown that the elevation of the glucose absorption rate was not consequent to a greater delivery of sugar into the intestines, but rather to the absorption of a greater proportion of that sugar in contact with the intestinal mucosa. These considerations suggest that a mechanism other than simple diffusion, capable of selectively enhancing the glucose absorption gradient, is rapidly developing just after hatching.

The phlorhizin-induced depression of glucose (but not sorbose) absorption here seen in the chick is similar to findings with other experimental animals(3). This effect occurred at all ages including 0-day-old chicks where sorbose and glucose absorption rates were quantitatively similar. Even at this early age, then, there is some basis for believing that the mode of absorption of these two sugars differs. Possibly the differentiative processes underlying the changing capacity to absorb glucose between 0 and 2 days of age are already under way at hatching. Lending credence to this view, the work of Moog(7) has shown that enzymes in the chick duode-

TABLE I. Results of Absorption Experiments.

Age in days	No. of chicks	Treatment*	Absorption coefficient (mg/100 g body wt/30 min.)	Avg % of sugar administered (a) Recov'd above intestines	(b) Recov'd from intestines	(c) Absorbed	(d) Delivered into intestines (b + c)
0	10	Glucose	49 ± 15§	21	62	17	79
	15	Glu + P†	22 ± 11	17	75	7	82
	12	Sorbose	38 ± 11	13	74	14	88
	12	Sorb + P	31 ± 9	16	73	11	84
2	10	Glucose	184 ± 34	13	23	64	87
	12	Glu + P	65 ± 21	10	67	24	91
	12	Sorbose	48 ± 7	16	67	17	84
	11	Sorb + P	48 ± 12	16	67	17	84
3	11	Glucose	192 ± 44	15	19	67	86
	10	Glu + P	76 ± 16	21	52	27	79
4	12	Glucose	181 ± 29	16	19	65	84
	22	Glu + P	80 ± 28	19	52	29	81
	11	Sorbose	53 ± 15	28	53	19	72
	11	Sorb + P	53 ± 15	17	64	19	83
8-10‡	31	Glucose	213 ± 40	17	8	75	83
	32	Glu + P	103 ± 30	12	52	36	88
	12	Sorbose	59 ± 13	26	53	22	75
	12	Sorb + P	58 ± 14	20	59	21	80

* Avg dose range of glucose or sorbose was 27-29 mg/10 g body wt.
† P = phlorhizin added to sugar solution for final concentration of 0.002 M.
‡ Data from chicks 8-10 days old were pooled since there were no significant differences from one age to the next.
§ Mean ± S.D.

num, thought to be concerned with absorption, are rapidly increasing at hatching but do not attain maximal activity until about 2 days thereafter.

Summary. 1. Absorption rates of glucose and sorbose, alone and with phlorhizin, were determined in chicks between 0 and 10 days of age. 2. Sorbose fulfilled the expectations of a passively absorbed sugar in that its absorption velocity was uniformly slow and uninfluenced by phlorhizin at all ages. 3. Glucose absorption velocity increased to a level 4 times as rapid as sorbose within 2 days of hatching and was inhibited by phlorhizin in all age groups. 4. These data, in support of earlier findings(1), suggest that the mechanism(s) responsible for the selective absorption of glucose is not fully developed at hatching, but rapidly achieves an adult level of function within 2 days thereafter.

1. Bogner, P. H., PROC. SOC. EXP. BIOL. AND MED., 1961, v , .
2. Verzár, F., *Biochem. Z.*, 1935, v276, 17.
3. Crane, R. K., *Physiol. Rev.*, 1960, v40, 789.
4. Cori, C. F., *J. Biol. Chem.*, 1925, v66, 691.
5. Golden, W. R. C., Long, C. N. H., *Am. J. Physiol.*, 1942, v136, 244.
6. Somogyi, M., *J. Biol. Chem.*, 1945, v160, 61.
7. Moog, F., *J. Exp. Zool.*, 1950, v115, 109.

Received January 30, 1961. P.S.E.B.M., 1961, v107.

Effects of Adrenalectomy, Thyroidectomy, Thyro-adrenalectomy and Cortisone on Eruption Rate of Incisors in Adult Female Rats.* (26598)

L. V. Domm and W. A. Wellband†
Department of Anatomy, Stritch School of Medicine, Loyola University, Chicago, Ill.

It has been known that the rate of eruption of the continuously erupting incisor in rats is severely retarded following hypophysectomy(1,2). Growth hormone(1,3) was observed to be ineffective, whereas thyroxin (3) and cortisone(4) were effective in restoring the rate in hypophysectomized rats and in accelerating it in normal rats(4,6,7). Thyroidectomy(8), propylthiouracil treatment (5), and adrenalectomy(4,7) were found to result in a decrease in eruption rate although not so severe as that observed following hypophysectomy. The severity of retardation in eruption rate noted in thyroidectomized rats was observed to be accentuated by a lack of salt in the diet(9). Parathyroidectomy and administration of parathyroid extract had no effect on eruption rate of incisors cut out of occlusal contact(10). The parathyroids are believed to have no effect on incisor eruption rate(11).

There is considerable evidence to indicate that the adrenals and the thyroids play an important role in the process of eruption of the incisors but there is little information to suggest the nature of this role. The present experiments were designed to determine and compare the effects of adrenalectomy, thyroidectomy, and thyro-adrenalectomy, with or without cortisone‡ treatment, on rate of eruption of incisors in young adult rats.

Procedures. Weekly observations were made on eruption rates of maxillary incisors in 28 thyro-parathyroidectomized and 21 intact, female, Sprague-Dawley, albino rats, beginning age 90 days. After 8 weeks, 16 of the thyroidectomized and 11 of the normals were adrenalectomized and maintained on 1% NaCl in the drinking water and observations continued for a second 8 week period. At the end of the second period, each of the 4 groups was subdivided into a treated and a control group and the experiment continued for a third period of 6 weeks. Treated rats received daily injections of 1.5 mg cortisone and were maintained on tap water while controls were continued without change. The experiment was terminated at the end of the sixth week of period III.

All animals were maintained on a diet of Purina Dog Chow pellets and tap water or tap water with NaCl *ad libitum*. Weekly eruption rates of upper incisors were determined by a method previously described (8, 11). Weekly averages for the first 16 weeks were analyzed by means of the t-test. The F-test was utilized in analyzing averages for each of the 3 periods and to make intergroup comparisons. Source of error used in the F-test was the "within group Mean Square."

Three of the 11 adrenalectomized and 5 of the 15 thyro-adrenalectomized rats were found to have adrenal rests following histological examination of suspicious tissue found on post-mortem. Comparison of eruption rates in adrenalectomized rats possessing adrenal rests, with those of completely adrenalectomized rats, revealed a significant difference but there was no significant difference in response of eruption rates to cortisone treatment. Consequently, weekly eruption rates of adrenalectomized rats possessing adrenal rests on post-mortem were not included in the calculation of averages, excepting those of the 2 cortisone-treated groups.

Serial sections of the thyroid region of tracheas of thyroidectomized rats were examined, and of 20 such examinations considered as adequate, 9 were found to have microscopic rests of thyroid tissue. Evaluation of individual incisor growth rates

* Supported in part by U.S.P.H.S. grant.

† Predoctoral Fellow U.S.P.H.S.

‡ Cortisone (Cortone Acetate) generously supplied by Merck, Sharp & Dohme, Division of Merck & Co., Inc., Philadelphia, Pa.

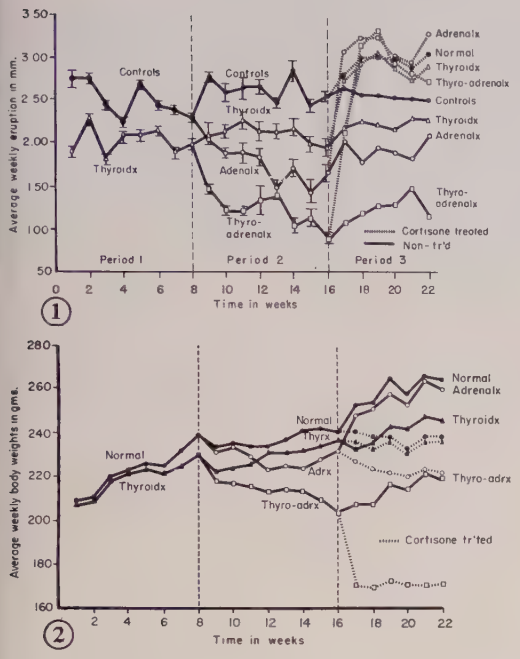


FIG. 1. Avg weekly eruption rates of maxillary incisors in thyroidectomized, adrenalectomized and thyro-adrenalectomized rats with and without cortisone administration. Vertical lines represent the stand. error of the mean.

FIG. 2. Effects of thyroidectomy, adrenalectomy, thyro-adrenalectomy and cortisone administration on body wt.

showed no consistent correlation between the presence or absence of thyroid rests and the eruption rates in many of the animals. Due to the difficulty of assessing the functional capacity of this tissue, it was decided not to exclude any of the thyroidectomized rats from the data, but to report averages for the total group and for the group determined complete by post-mortem histological examination.

Results. The data are summarized in Fig. 1. Thyroidectomy resulted in a highly significant ($P = .001$) reduction of 30% in eruption rates by the end of the first week. Rates showed considerable fluctuation during the first 4 weeks but thereafter tended to remain fairly constant at approximately 20% below normal. Differences between groups proved to be highly significant for each week except the fourth, when eruption rates of normal controls decreased temporarily. The difference observed at this time was not significant ($P = > .05$). The average eruption rate for the first 8 weeks was $2.03 \pm .37$ mm§ (224 observations, $P = .001$), 19% below that of normal controls whose average was $2.51 \pm .32$ mm (168 observations). The average eruption rate during this period for 9 thyroidectomized rats possessing thyroid rests was $2.12 \pm .38$ mm and for 11 believed to be complete $1.91 \pm .37$ mm (24% reduction). The differences between means of these 2 groups, although small, was found to be highly significant, as were differences between the averages of each of the groups and the normal controls.

During the second 8-week period eruption rates of thyroidectomized rats continued to maintain a fairly constant level at approximately 18% below normal. The average for the second period was $2.15 \pm .36$ mm (96 observations, $P = .001$) as compared with $2.63 \pm .30$ mm (80 observations) for controls. Weekly averages of 7 thyroidectomized rats, verified as complete, showed essentially the same pattern. The average of this group for the second period was 2.02 mm (23% reduction). Adrenalectomy alone at this time, resulted in a highly significant decrease in rate (25%) by the end of the first week. The decrease became progressively more pronounced during the following 7 weeks but thereafter was less pronounced. Average rate for the second period was $1.75 \pm .33$ mm (64 observations, $P = .001$), 33% below that of normal controls. When adrenalectomy was superimposed on thyroidectomy, eruption rates became progressively more depressed, to 65% below normal by the eighth week post-operatively. The average 8-week eruption rate was $1.23 \pm .30$ mm (64 observations, $P = .001$), 53% below normal. All weekly comparisons between normal controls and each of the operated groups were significant ($P =$ or $< .05$). The presence or absence of thyroid rests apparently had little effect on degree of reduction following the second operation (adrenalectomy) since rats possessing thyroid rests showed the same, or a more severe, reduction than those verified as complete.

§ Standard deviation

TABLE I. Summary of Effects on Eruption Rates during Period III of Experiment.

Groups	Mean	S.D.*	% of normal	No. of observations
Normal	2.57	.21	100	30
" + cortisone	2.97	.23	116	30
Adrenalectomy	1.95	.19	76	26
" + cortisone	3.16	.36	123	24
Thyroidectomy	2.25	.20	88	36
" + cortisone	2.89	.24	113	36
Thyro-adrenalectomy	1.26	.27	49	24
" + cortisone	2.86	.41	111	30

* Stand. dev.

In comparing the 8-week averages for period II, the sum of the reductions noted, in thyroidectomized and adrenalectomized groups, was 51% which closely approximated the actual reduction (53%) observed in the thyro-adrenalectomized group. The pooled variation of the thyro-adrenalectomized and normal groups was compared with the pooled variation of the adrenalectomized and thyroidectomized groups utilizing the F-test. The difference was not significant ($F = < 1$), which was interpreted to indicate that there had been no interaction and that the effects of the combined operations equaled the sum of the effects of each operation. Additional comparisons showed a highly significant ($P = .001$) difference between each of the operated groups.

The results for period III are summarized in Table I. During period III eruption rates of the 3 non-treated surgical groups showed a slight increase over previous rates for period II, while rates of the normal controls remained about the same. Administration of cortisone resulted in highly significant increases in eruption rates of all 4 groups to levels above that of non-treated intact rats. The cortisone-treated, adrenalectomized, group showed significantly greater increases than any of the other cortisone-treated groups. There were no significant differences between the responses of the other 3 groups during this period.

Weekly body weights of thyroidectomized and adrenalectomized rats (Fig. 2) fluctuated within a narrow range, somewhat below that of normals. Average weights of thyroidectomized and adrenalectomized rats were respectively 1% and 3% below those of normal controls by the end of period II and 7% and 1% at the end of period III. Removal of both glands resulted in a progressive decrease in body weight so that averages were 15% and 16% below those of controls by the end of periods II and III respectively. Cortisone administration resulted in weight losses in all 4 groups which averaged 10% in thyroidectomized and intact rats, 16% in adrenalectomized and 35% in thyro-adrenalectomized by the end of period III.

Adrenal weights of cortisone-treated, intact rats (Avg. 43.8 $\pm$ 4.4§ mg) were reduced to approximately the same degree as those of thyroidectomized rats (Av. 44.3 $\pm$ 4.6 mg). Cortisone treatment in thyroidectomized rats brought about further reductions in adrenal weights (Av. 33.5 $\pm$ 6.5 mg). The average weight of adrenals in normal controls was 65.3 $\pm$ 8.2 mg.

The small number of rats used in each group of period III did not permit adequate evaluation of the effects of thyroid rests; however, they appeared to have little effect on eruption rates. Adrenals tended to be slightly heavier in non-treated, thyroidectomized rats possessing thyroid rests.

Discussion. Close agreement was observed between average eruption rate of normals in each of the 3 periods and normal averages previously reported(2,7). The 33% reduction in eruption rate observed following adrenalectomy closely approximates the 31% reduction reported by us for young rats (7) and the 31% reported by others(4). However, the reduction in rate following thyroidectomy, either during the early

post-operative period or throughout the experiment, or in rats with no discernible thyroid tissue, was not so severe as the reduction previously reported by others following thyroidectomy at birth (45%)(8), or following administration of a "thyroid-blocking" agent (48%)(5). While age could account for the difference in rate in the first instance it does not apply in the second. Whether different strains have different rates has not been determined.

In the present experiments, evaluation of the thyroid status of the animals was difficult. We were unable to find any substantial effect, due to presence or absence of thyroid rests, on incisor eruption rate or body weight. Differences were usually small and inconsistent. Observations have been reported(12) to indicate that there is a decrease in the thyroxin requirements of older animals. Preliminary observations on immature, thyroidectomized rats have shown a more severe reduction in incisor eruption rate than that observed in the present older series which would seem to provide support for the above observation.

We may tentatively conclude that where adrenalectomy is superimposed on thyroidectomy, in young adults, the effects are additive. The resulting cumulative depression in eruption rates closely approximated that previously reported(2) a few weeks after hypophysectomy. However, our thyro-adrenalectomized rats did not show the progressive depression in eruption rate following the eighth week that has been reported by others following hypophysectomy(1,2). Whether the presence of hidden thyroid remnants, age of the animals, or other factors are responsible for this difference in response remains to be determined. The results suggest that the severe depression in incisor eruption rate reported following hypophysectomy(1,2) may be the result of a reduction in available thyroid and adrenal hormones.

In earlier experiments(7) adrenalectomy in young rats brought about little change in body weight during the first few weeks following operation, a period during which eruption rates were severely depressed. The present observations following endocrine removal might be interpreted as indicating a correlation between weight loss and reduction in eruption rate. However, cortisone administration in these animals resulted in weight losses in all groups, yet despite this loss significant increases in eruption rates were observed. Thyroxin(3) and desiccated thyroid (5) administration also stimulate incisor eruption rates yet desiccated thyroid and cortisone have been reported(13) to be "antagonistic" in their effects on food consumption and body weight in intact rats.

That body weight cannot be considered as a significant factor in regulation of the incisor eruption rate seems indicated from the above results as well as from earlier work(14) where cortisone administration brought about precocious eruption of incisors in new born rats despite severe weight loss. Preliminary observations on dietary restriction, in which severe weight loss was observed, have shown no decrease in eruption rate, verifying the observations of others(10).

The manner in which cortisone acts on eruption rate remains to be determined. Histological and histochemical studies may clarify some of these problems.

Summary. The eruption rate of maxillary incisors was determined in normal, thyroidectomized, adrenalectomized and thyro-adrenalectomized, adult, female rats, with or without daily administration of cortisone. Thyroidectomy resulted in an average reduction in rate of 19% during the first 8 weeks. When adrenalectomy was superimposed on thyroidectomy 8 weeks post-operatively, average rate decreased to 53% below normals, indicating a cumulative effect. Adrenalectomy alone at this time resulted in a 33% average decrease. Administration of cortisone increased the rates in all 4 groups to levels above that of normal controls. The results suggest that the normal rate of eruption of the incisor may be influenced by a synergistic action of the thyroid and adrenal hormones.

1. Schour, I., Van Dyke, H. B., PROC. SOC. EXP. BIOL. AND MED., 1932, v29, 378.

2. Baume, L. J., Becks, H., Ray, J. C., Evans, H. M., *J. Dent. Res.*, 1954, v33, 91.

3. Baume, L. J., Becks, H., Evans, H. M., *ibid.*, 1954, v33, 104.

4. Garren, L., Greep, R. O., *Endocrinol.*, 1960, v66, 625.

5. ———, PROC. SOC. EXP. BIOL. AND MED. 1955, v90, 652.

6. Domm, L. V., Marzano, R., *Anat. Rec.*, 1954, v118, 383.

7. Domm, L. V., Wellband, W. A., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 582.

8. Baume, L. J., Becks, H., Evans, H. M., *J. Dent. Res.*, 1954, v33, 80.

9. Frey, B. J., Genitis, V., Schour, I., Templeton, R. D., *Am. J. Physiol.*, 1936, v116, 53.

10. Bryer, L. W., *Intern. Dent. J.*, 1957, v7, 432.

11. Schour, I., Massler, M., *The Rat in Laboratory Investigation,* Lippincott, Philadelphia, 1949, Chap. 6.

12. Fleischmann, W., *Comparative Physiology of the Thyroid and Parathyroid Glands,* Charles C. Thomas, Springfield, Ill., 1951, p. 30.

13. Greenberg, S. M., Aterman, K., *Metab. Clin. & Exp.*, 1955, v4, 264.

14. Parmer, L. G., Katonah, F., Angrist, A. A., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 215.

Received February 6, 1961. P.S.E.B.M., 1961, v107.

A Neuropharmacological Investigation of the Convulsant Action of 4-Phenyl-4-Formyl-N-Methyl Piperidine (1762 IS)* (26599)

V. G. LONGO AND A. PINTO CORRADO (Introduced by F. W. Schueler)
Laboratorio di Chimica Terapeutica, Istituto Superiore di Sanità, Rome, Italy

During an investigation of a series of compounds synthetized for possible strychnine-like activity, one in particular, 4-phenyl-4-formyl-N-methyl-piperidine (1762 IS), was found to possess marked activity as indicated by preliminary screening(1).

In the present work, a more detailed analysis of the action of 1762 IS was made. The usual pharmacological procedures were used as well as more specific technics which included a study of the modifications of the electrical activity at the various levels of the cerebro-spinal axis, the effects of application of the substance to the cerebral and cerebellar cortex on the cortical electrical activity and its action on spinal integratory mechanisms.

Material and methods. Acute toxicity was determined by intraperitoneal and oral administration in mice and intravenously in rabbits. The LD_{50} was determined in mice using the probit method.

Eleven unanesthetized rabbits paralyzed with 5 mg/kg of gallamine intravenously were used for registration of the cortical and subcortical electrical activity according to a method described previously(2). In all animals arterial blood pressure was simultaneously recorded. Histological sections were prepared routinely to verify the exact location of the electrodes in the subcortical structures. Experiments in which the compound was applied topically were carried out in 8 rabbits unanesthetized and without neuromuscular paralysis. Disks soaked in various concentrations of the drug were applied on the sensorimotor area of the cortex and on the *lobulus medianus* of the cerebellum. Freshly made water solutions of the hydrochloride salt were used; the dosage is given in weight of the free base.

Experiments on the action of the drug upon the spinal cord were carried out in spinal (Th-10) cats lightly anesthetized with chloralose. After lumbar laminectomy, the appropriate ventral roots were cut and mounted on silver electrodes for recording purposes. The spinal cord was covered with warm paraffin oil. Mono- and polysynaptic reflexes were evoked by stimulation of the popliteal branch of the sciatic nerve. In the experiments dealing with the "primary" inhibition, a maximal monosynaptic reflex was evoked by stimulation of the central stump of the biceps and semi-tendinous nerves (BST). Inhibition of this reflex was obtained by stimulating the group Ia afferent fibers of the quadriceps

* This research has been sponsored in part by the Air Research and Development Command, USAF, through its European Office.

nerve (Q). Further details of the technical procedure have been described previously (3).

Results. Acute toxicity. The LD_{50} in mice was found to be 42 mg/kg intraperitoneally and 284 mg/kg perorally. Mice injected with sublethal doses showed continuous running movements, elevation of the tail (Straub reaction), twitching and jerking. Death of the animals was preceded by muscular tremors and a tetanic spasm. In rabbits, doses of 1-2 mg/kg *i.v.* were found to bring about hyperreflexia, spasmodic continuous jerking and, in some instances, tetanic convulsions. Higher doses (3 mg/kg) provoked, in all animals, the characteristic tonic attack with opisthotonus and extension of the hind legs, which was normally followed by death. In those cases where the animal survived the initial attack, it was possible to elicit new attacks by a variety of external stimuli, *e.g.*, acoustical or visual and especially by touching the back of the animal.

Electroencephalogram. Doses of 1 to 2 mg/kg (which cause hyperreflexia in animals not treated with gallamine) result in a desynchronization of the tracing, characterized by an increase in frequency and a decrease in amplitude of the cortical waves, and by appearance of a regular 5 c/sec rhythm in the thalamic lead. The tracing of the electrospinogram, however, shows no change. Doses of 3 mg/kg bring about the appearance of a characteristic seizure consisting of 20-30 c/sec waves in the spinal, cerebellar and mesencephalic leads (Fig. 1). Concurrently, only desynchronization is noticed in the cerebral cortex. The thalamic lead exhibits synchronous and regular waves at 8-9 c/sec. In 4 out of 7 animals treated with higher doses (4-5 mg/kg) the spinocerebellar seizure developed into a "grand mal" EEG pattern in all leads.

An increase in blood pressure accompanied the alterations of the EEG and became particularly noticeable during the spino-cerebellar seizures. These seizures lasted for several minutes and were frequently interrupted by short periods of elecrtical silence accompanied by abrupt drops in blood pressure.

Topical application. At a concentration of 0.1%, 1762 IS applied to the cortex induced the appearance of spikes, at first in the region of the treated zone and thereafter spreading to the entire cortex. Spreading of the spik-

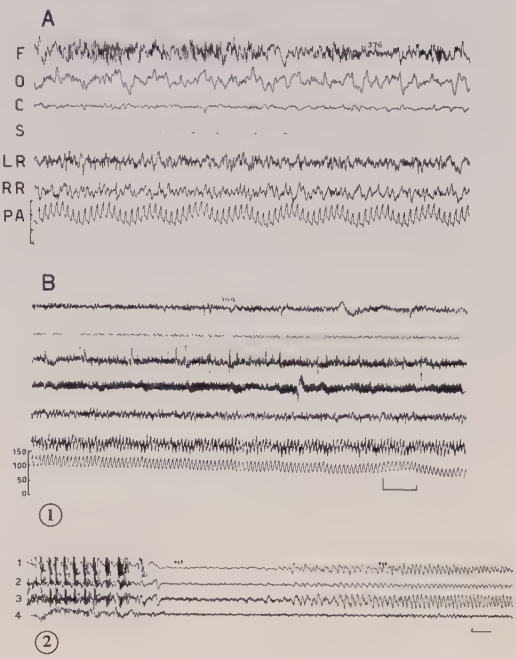


FIG. 1. A. Control tracing. Spindles are present in the sensorimotor cortex. No activity is registered from the spinal cord lead. B. Five min. after i.v. administration of 3 mg/kg 1762 IS. A sustained seizure, characterized by spikes at 20-30 c/sec., appears in the spinal, cerebellar and reticular leads; concurrently, there is desynchronization of the cortical tracing.

Unanesthetized rabbit treated with gallamine. Leads. F: anterior sensorimotor cortex; O: optic cortex; C: *Lobulus medianus* of cerebellum; S: lumbar enlargement of spinal cord; LR: left reticular midbrain formation; RR: right reticular midbrain formation; PA: femoral blood pressure.

Calibration: horizontal bar, 2 sec.; the left vertical bar indicates 100 μV for cerebellar and spinal tracing; the right vertical bar indicates 100 μV for the other leads.

FIG. 2. Convulsant action of 1762 IS applied topically to the right sensorimotor cortex of the rabbit. The tracing was recorded 15 min. after application of 1762 IS 0.1%. Clonus of the snout and movements of vibrissae were seen simultaneously with appearance of the burst of spikes. Few seconds after the seizure, waves of high voltage at 2 c/sec. appear in the record.

Unanesthetized rabbit without gallamine. Leads. 1: R anterior sensorimotor cortex – L anterior sensorimotor cortex; 2: L anterior sensorimotor cortex – L posterior sensorimotor cortex; 3: R posterior sensorimotor cortex – L posterior sensorimotor cortex; 4: R optic cortex – L optic cortex.

Calibration: 50 μV, 2 sec.

ing activity was consistently provoked by stimulation of the reflexogenic zones, such as the muzzle and the vibrissae of the opposite side when the drug was applied to the anterior sensorimotor cortex. Immediately after the seizures, there appears an unusual EEG picture consisting of waves of high voltage and of a frequency of 2 c/sec in all leads (Fig. 2). However, topical application (1%) on the cerebellum did not cause the appearance of cerebellar spikes.

Mono and polysynaptic reflexes and spinal "primary" inhibition. The influence of increasing doses of 1762 IS on the segmental reflexes was observed in 4 spinal cats. At a dose of 2-3 mg/kg the polysynaptic wave increased both in duration and in height, whereas the monosynaptic spike remained unchanged or was slightly reduced in amplitude. Higher doses (up to 5 mg/kg) further enhanced the polysynaptic component. Simultaneously, there was a more marked decrease of the monosynaptic reflex (to 60-70% of control).

The effect of 1762 IS on the spinal "primary" inhibition was studied in 3 animals. Three to 4 mg/kg blocked the inhibition of the BST monosynaptic reflex due to stimulation of the Q nerve. Concomitant with this effect, a slight decrease of the monosynaptic BST reflex occurred.

Discussion. These results have demonstrated that 1762 IS can be considered as another synthetic drug possessing strychnine-like properties. There exist, however, some slight differences between strychnine and 1762 IS which, even though not observed during toxicity studies in various laboratory animals, are evident from the results of electrophysiological procedures. From the point of view of its action on cerebral electrical activity, it has been noticed that, after the spino-cerebellar-mesencephalic seizure (which is characteristic of strychnine also), in about 50% of the treated animals, a "grand mal" EEG pattern developed in all leads. This picture is only rarely observed with strychnine(2,4). Also, a difference was found between the action of 1762 IS and of the alkaloid on the spinal cord. In previous investigations, using the same experimental conditions(2), both strychnine and a synthetic strychnine-like compound, 5,7-diphenyl-1,3-diazadamantan-6-ol, consistently provoked an enhancement of the monosynaptic reflex. On the contrary, 1762 IS causes a slight decrease of this reflex. This indicates that the block of the spinal "primary" inhibition can occur independently of enhancement of the monosynaptic reflex. Thus, it may be concluded that 1762 IS has a specific depressant action on the monosynaptic excitatory arch. or that it is inconsistent in its influence on the various spinal inhibitory systems.

These results indicate a strong similarity between 1762 IS and thebaine, the action of which, on the EEG and on spinal primary inhibition, has been described recently by Corrado and Longo(5). In this connection, it is of considerable interest that 1762 IS is the aldehyde corresponding to the 1-methyl-4-phenyl isonipecotic acid, whose ethyl ester is meperidine, which possesses significant analgesic properties. A marked structural similarity exists also between morphine and thebaine. With both pairs of drugs these modifications in structure lead from a predominance of analgesic activity to a predominance of convulsant strychnine-like activity.

Summary. In preliminary toxicity studies, a synthetic compound, 4-phenyl-4-formyl-N-methyl-piperidine (1762 IS) showed convulsant properties very similar to those of strychnine. More detailed neuropharmacological investigations dealing with the action of 1762 IS on cerebral electrical activity, on the mono- and polysynaptic reflexes and on the "primary" spinal inhibition have confirmed the similarities between this compound and strychnine. Some relationships between chemical structure and pharmacological properties are discussed.

1. Chiavarelli, S., Iorio, M. A., Settimj, G., Marini-Bettolo, G. B., *Gazz. Chim. Ital.,* 1957, v87, 427.

2. Longo, V. G., Silvestrini, B., Bovet, D., *J. Pharmacol. & Exp. Ther.,* 1959, v126, 41.

3. Longo, V. G., *ibid.,* 1961, v132, 240.

4. Johnson, B., *J. Neurophysiol.,* 1955, v18, 189.

5. Corrado, A. P., Longo, V. G., *Arch. Int. Pharmacodyn.,* 1961, in press.

Received February 13, 1961. P.S.E.B.M., 1961, v107.

Hexosamine and Hydroxyproline Contents of Tissues in Scurvy.* (26600)

Sachchidananda Banerjee and Pulak Kumar Ghosh
Depts. of Physiology and Biochemistry, Bikaner Medical College, Bikaner, India

Although the formation of connective tissue is defective in scurvy(1,2) the role of ascorbic acid in the process is not clear. The possible metabolic defect in ascorbic acid deficiency might be the inability of the tissues to hydroxylate proline to form hydroxyproline(3) and incorporation of an "active" hydroxyproline into collagen(4). The view, however, is contradictory to the observation of Mitoma and Smith(5) that hydroxylation of proline is not decreased by L-ascorbic acid deficiency in guinea pigs. According to them L-ascorbic acid possibly participates in the maturation of fibroblasts and thus exerts an indirect effect on collagen synthesis. A deficiency of L-ascorbic acid in guinea pigs either interferes with the synthesis of new collagen or results in its destruction as it is produced(6). There is a decrease in the activity of oxidative enzymes(7-9) in scorbutic guinea pigs. The generalised lowered cellular metabolic activities might also result in defective production of matrix. Collagen which is a basic building material of connective tissue is a protein. Insulin is involved in biosynthesis of proteins(10). Scorbutic guinea pigs suffer from hypoinsulinism(11-13). It is therefore possible that insulin deficiency associated with scurvy might interfere with formation of collagen. Hexosamine and hydroxyproline which are respectively the principal constituents of the ground substance and the collagen fibers of connective tissues, therefore, were estimated in the different tissues of normal, scorbutic and insulin-treated scorbutic guinea pigs.

Experimental. Male guinea pigs weighing from 250 to 300 g were used. Selection of the animals, feeding them a scorbutogenic diet(14), separation of the animals into groups containing one normal, one scorbutic and one insulin-treated scorbutic guinea pig and injection of insulin into the animals intended for insulin treatment were the same as described previously(15). On the fourth week of the experiment all the animals were killed by decapitation and portions of liver, kidney, bone, cartilage, aorta, lung, skeletal muscle and skin were analyzed for hexosamine and hydroxyproline. The tissues were dried, defatted and hydrolyzed by the method of Blix(16). The hydrolysate was used for estimation of hexosamine(16) and hydroxyproline(17).

Results. The results are given in Tables I and II. Hexosamine content of aorta, kidney, skeletal muscle and bone was increased significantly in scorbutic guinea pigs. Lung, liver, cartilage and skin of the animals under different treatments did not show any change in hexosamine content. Prolonged insulin treatment of the scorbutic animals did not influence the hexosamine content of the tissues studied. The hydroxyproline content of the cartilage and kidney decreased significantly in the scorbutic guinea pigs. The other tissues studied did not show change from normal. Prolonged insulin treatment, however, not only enhanced the hydroxyproline of cartilage and kidney but also those of lung and skin.

Discussion. In a number of tissues of scorbutic guinea pigs hexosamine, which is the principal constituent of the ground substance of connective tissue, was significantly increased in comparison to normal controls. This might be due to increased biogenesis or decreased catabolism of hexosamine in scurvy. Insulin treatment of scorbutic animals did not alter the hexosamine content of tissues, evidently indicating that insulin had no role in the process. According to Dorfman and Schiller(18) insulin deficiency adversely affects the synthesis of mucopolysaccharides. This concept, however, may not be applicable to connective tissue metabolism in scorbutic guinea pigs. Ascorbic acid possibly plays a direct role in the process.

* The Indian Council of Medical Research financed this research project.

TABLE I. Hexosamine Content of Tissues of Guinea Pigs. Values are g/100 g of dry tissue.

Tissue	Condition of animal		
	Normal	Scorbutic	Insulin-treated scorbutic
Aorta	.64 ±.045	1.14 ±.067	1.31 ±.11
Lung	.68 ±.03	.70 ±.02	.69 ±.03
Liver	.47 ±.02	.47 ±.06	.47 ±.03
Skin	.50 ±.03	.48 ±.04	.49 ±.03
Cartilage	1.81 ±.14	2.71 ±.20	2.86 ±.16
Kidney	.24 ±.007	.81 ±.06	.76 ±.04
Skeletal muscle	.34 ±.02	.66 ±.047	.62 ±.06
Bone	.39 ±.026	.67 ±.019	.67 ±.038

Values are mean ± stand. error. Sixteen animals in each group.

In a few tissues of the scorbutic animals hydroxyproline, which is the principal constituent of collagen, was decreased considerably and after prolonged insulin treatment of these animals, hydroxyproline content of the tissues was increased. The interference with collagen synthesis in some tissues, therefore, might be due to a lack of insulin which results from scurvy(11-13).

It is probable that the hydroxylation of proline for transformation of precollagen to mature collagen is dependent on insulin. In the relative insufficiency of the hormone in the scorbutic condition the mature collagen fibers are not properly formed, leading to disorganization of the connective tissue. The ground substance freed from the binding force of the proliferating strands of collagen fibers depolymerizes and the mucopolysaccharides eventually find their way into the circulation. This causes an increase in the serum level of mucoproteins in scorbutic guinea pigs as reported earlier(19).

TABLE II. Hydroxyproline Content of Tissues of Guinea Pigs. Values are g/100 g dry tissue.

Tissue	Condition of animal		
	Normal	Scorbutic	Insulin-treated scorbutic
Aorta	3.67 ±.18	3.79 ±.22	4.15 ±.13
Lung	1.35 ±.05	1.38 ±.04	2.29 ±.23
Liver	.36 ±.02	.38 ±.03	.42 ±.03
Skin	4.86 ±.19	4.83 ±.20	7.52 ±.23
Cartilage	2.89 ±.19	1.14 ±.13	2.90 ±.13
Kidney	.74 ±.03	.36 ±.03	.69 ±.04
Skeletal muscle	1.23 ±.18	1.29 ±.16	1.61 ±.24
Bone	2.28 ±.12	2.30 ±.13	2.29 ±.14

Values are mean ± stand. error. Sixteen animals in each group.

Summary. The hexosamine and hydroxyproline contents of aorta, lung, skin, kidney, skeletal muscle, bone and cartilage were determined in normal, scorbutic and insulin-treated scorbutic guinea pigs. In the scorbutic guinea pigs hexosamine content significantly increased in aorta, kidney, skeletal muscle and bone and no change was observed in lung, liver, cartilage and skin. Insulin treatment had no effect on these contents. Hydroxyproline content decreased significantly only in kidney and cartilage of scorbutic guinea pigs. Prolonged insulin treatment of the scorbutic animals produced a significant increase in the hydroxyproline contents of cartilage, kidney, lung and skin.

The possible implications of the results have been discussed.

1. Follis, R. H., Jr., The Pathology of Nutritional Diseases, Blackwell, Oxford, 1948.

2. Wolbach, S. B., Bessey, O. A., *Physiol. Revs.*, 1942, v22, 233.

3. Gould, B. S., Woessner, T. F., *J. Biol. Chem.*, 1957, v226, 289.

4. Robertson, W. van B., Hiwett, J., Herman, C., *ibid.*, 1959, v234, 105.

5. Mitoma, C., Smith, T. E., *ibid.*, 1960, v235, 426.

6. Gross, J., *J. Exp. Med.*, 1959, v109, 557.

7. King, C. G., *J. Am. Med. Assn.*, 1950, v142, 563.

8. Hill, C. R., Bourne, G. H., *Brit. J. Nutrition*, 1954, v13, x.

9. Banerjee, S., Biswas, D. K., Singh, H. D., *J. Biol. Chem.*, 1959, v234, 405.

10. Bouckaert, J. P., deDuve, C., *Physiol. Revs.*, 1947, v27, 39.

11. Banerjee, S., *Nature* (London), 1944, v153, 344.

12. Banerjee, S., Ghosh, N. C., *J. Biol. Chem.*, 1947, v168, 207.

13. Banerjee, S., Biswas, D. K., Singh, H. D., *ibid.*, 1958, v230, 261.

14. Banerjee, S., *ibid.*, 1945, v159, 327.

15. Banerjee S., Biswas, D. K., *ibid.*, 1959, v234, 3094.

16. Blix, G., *Acta Chem. Scand.*, 1948, v2, 467.

17. Martin, C. J., Arnold, A. G., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 461.

18. Dorfman, A., Schiller, S., *Recent Progress in Hormone Research,* 1958, v14, 427.
19. Banerjee, S., Ghosh, P. K., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 63.

Received February 13, 1961. P.S.E.B.M., 1961, v107.

Incorporation of S^{35} from L-Methionine in Tumor-bearing and Protein-depleted Rats.* (26601)

ROBERT W. WANNEMACHER, JR. (Introduced by J. B. Allison)
Bureau of Biological Research, Rutgers University, New Brunswick, N. J.

Data have been presented to demonstrate an increase in the specific activity of certain plasma and tissue proteins in protein-depleted or tumor-bearing animals given S^{35} methionine. For example, Garrow found an increased uptake of S^{35} from labelled methionine in plasma proteins of depleted dogs(1) and malnourished children(2). Waterlow (3) reported a greater incorporation of S^{35} in the viscera of protein-depleted rats. Allison *et al.*(4) demonstrated an increased specific activity of serum proteins following an injection of S^{35} methionine into tumor-bearing or protein-depleted dogs. These and other data suggest a shift in the dynamic state of protein metabolism in depleted or tumor-bearing animals that could involve a number of cellular structures. The following experiments were performed to study the incorporation of S^{35} from labelled methionine into cellular proteins of liver and tumor tissues and into serum protein fractions of control, protein-depleted and tumor-bearing rats.

Methods. Male Wistar rats, 100-150 g, were divided into 3 groups of 30 animals each and placed on a diet containing 18% of casein as previously described by Allison *et al.* (5). On the first day of experiment a suspension of a Walker 256 carcinosarcoma was transplanted into one group, while on the seventh day another group was started on a protein-free diet. All animals were sacrificed after 17 days of experimentation. On day 7 of the experiment 10 rats from each group were placed in metal metabolism cages and daily food, urine and fecal collections were made for the next 10 days. The nitrogen concentration of the sample was determined by the micro-kjeldahl method.

At ½, 1, 2, 4, 6 and 12 hours before autopsy, 5 rats in each group were injected intraperitoneally with 0.2 mg of S^{35} L-methionine per kg of body weight. All rats were anesthetized with sodium pentobarbital (50 mg/kg) and sacrificed by exsanguination. The labelled tissue proteins were extracted and purified by the method of Zamecnik *et al.* (6). Liver and tumor tissues were fractionated into various cellular fractions by differential centrifugation. The nuclei were prepared in non-aqueous medium by the method of Carruthers *et al.*(7), and the mitochondria, microsomes, and cell sap were separated by a modification of the method of Hogeboom *et al.*(8). Serum was separated electrophoretically into various fractions by paper electrophoresis and dyed with alcoholic bromphenol blue, while total serum protein was determined by the copper sulfate falling drop method. All radioactive samples were counted in a windowless proportional flow counter and corrected for background, self-absorption, radioactive decay, back-scattering, geometry and body weight of the animals.

Results. The data in Table I demonstrate that the tumor-bearing rats were in positive nitrogen balance. If it is assumed that tumor tissue was essentially a "nitrogen trap", (9) then subtracting the tumor nitrogen from the total balance leaves the carcass (body minus tumor) near nitrogen equilibrium. During the same time period, the protein-depleted animals were in marked negative balance. These data are in agreement with these of Allison *et al.*(4) who reported a cor-

* Supported by grant from Am. Cancer Soc.

TABLE I. Nitrogen Balance in Tumor-Bearing, Protein-Depleted and Control Rats Fed a Diet Containing 18% Casein for 10 Days. Each value is the mean for 10 animals.

Diet	Nitrogen intake	Urinary nitrogen	Fecal nitrogen	Nitrogen balance	Tumor nitrogen	Carcass nitrogen bal. (nitrogen bal. – tumor nitrogen)
	g/kg					
18% casein (control)	11.18 ±.26*	2.94 ±.11	1.68 ±.14	+6.53 ±.21		
18% casein (tumor-bearing)	9.00 ±.18	3.51 ±.12	1.12 ±.13	+3.94 ±.22	3.03 ±.66	+.91 ±.88
P.F.D. (depleted)	0	.41 ±.16	.84 ±.04	−2.14 ±.06		

* Stand. error.

relation between decrease in serum albumin concentration and loss in body nitrogen in both tumor-bearing and protein-depleted dogs.

Fig. 1 shows that the specific activity of the serum proteins of the tumor-bearing group (open circles) was initially significantly (P less than 0.01) greater than either the protein-depleted (solid circles) or control animals (crossed circles), and that this activity fell rapidly to control levels by the end of 12 hours. Throughout the experiment, however, the protein-depleted rats had a higher specific activity than the controls. These results confirm the results of Garrow in the dog(1) and in human infants(2) and the conclusion of Allison *et al.*(4) that protein-depletion produces increased uptake of S^{35} methionine which can be correlated with degree of body nitrogen loss. The increased uptake of S^{35} by the tumor-bearing rat, however, could not be related to loss in body ni-

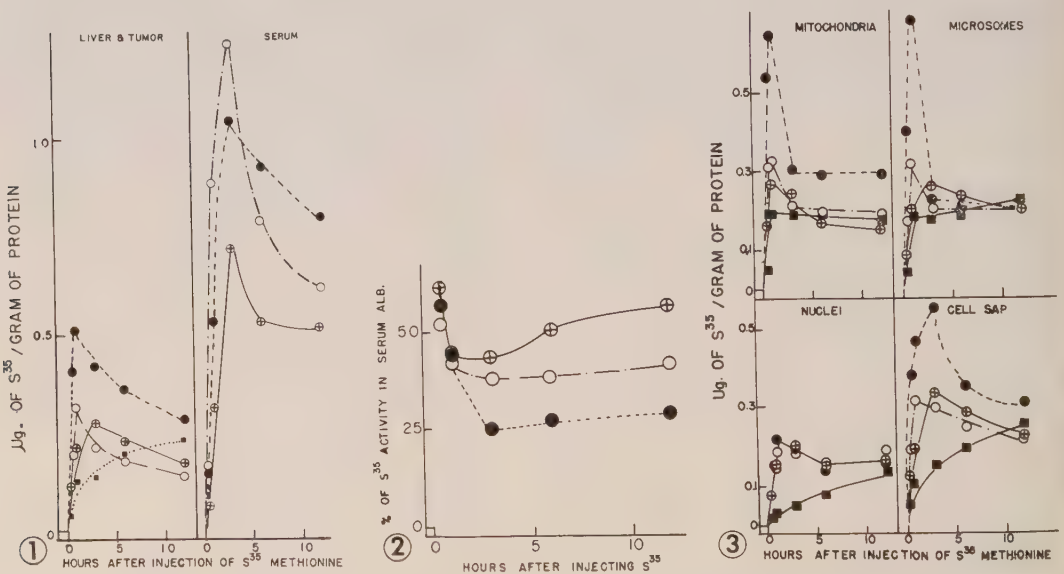


FIG. 1. Specific activity in μg of sulfur per g of protein of liver and tumor tissues and serum at various times after inj. of S^{35}-L-methionine. Each point is mean of 5 animals. Cross circles illustrate data using control animals, open circles tumor-bearing and closed circles protein-depleted rats. A square illustrates data from tumor tissue.

FIG. 2. Percent of total serum S^{35} activity in the albumin fraction at various times after inj. of S^{35}-L-methionine. Each point is mean of 5 animals. For significance of symbols see Fig. 1.

FIG. 3. Specific activity in μg of sulfur per g of protein of liver and tumor tissue of nuclei mitochondria, microsomes, and cell sap at various times after inj. of S^{35}-L-methionine. Each point is mean of 5 animals. For significance of symbols see Fig. 1.

trogen, results which are in agreement with those reported also for tumor-bearing dogs (4).

The average serum albumin concentrations were, control group, 2.72 ± 0.07, tumor-bearing 2.10 ± 0.10 and protein-depleted 1.77 ± 0.05 g per 100 cc. There was a lower percentage of total serum radioactivity in the albumin fraction in depleted rats than in the controls while the tumor-bearing animals fell in between (Fig. 2). These results can be interpreted to mean that a smaller amount of serum albumin was synthesized in depleted animals, and this decreased synthesis could be correlated with serum albumin concentration and body nitrogen loss.

Liver proteins of the depleted rats (Fig. 1 and 3) had a higher specific activity in the total proteins and in the mitochondria, microsomes and cell sap than was found in the control animals. Henderson *et al.*(10) and Thompson *et al.*(11) found that when rats were placed on a protein-free diet there was a decrease in serum and liver non-protein methionine. The concentration of S^{35} methionine would be relatively greater in an amino acid pool which was low in non-labelled methionine, and thus, a higher specific activity would be expected in the proteins synthesized from this pool. This concept, emphasized by Garrow(1,2), can explain the results found in serum and liver of protein-depleted rats. The liver nuclear proteins of the depleted animals, however, had a similar specific activity to that found in the control group. Because of the smaller amino acid pool, one would expect to find the values to be higher in the nuclear fraction of the liver in depleted rats. Therefore, it is possible that synthesis of liver nuclear proteins was decreased in the depleted animals. The specific activity of liver protein in tumor-bearing animals on the other hand, decreased below the values found for control rats 2 hours after injection of isotopic methionine. Subjecting these livers to differential centrifugation revealed (Fig. 3) that protein in nuclei, mitochondria, and microsomes had approximately the same specific activity as found in the control animals, while the cell sap fraction was initially higher in activity in the tumor-bearing animals and then fell below control values. As the specific activity of the liver protein (Fig. 1) of the tumor-bearing animals decreased, there was an increase in serum protein activity which then decreased as the tumor tissue (Squares with dotted line) continued to gain in isotopic activity. The data of several workers(12) indicated that tumor tissue has a preference for serum proteins as a source of amino acid for protein synthesis. It is possible that the tumor stimulated the liver to increase the synthesis of serum proteins which were then used by tumor tissue as source of amino acid for its rapid growth rate.

The nuclear proteins of the tumor tissue (Fig. 3) continued to increase in activity even 12 hours after injection of radioactive methionine. This fraction represented 40% of total tissue activity while in the liver it was 10% of total isotope concentration. Busch *et al.*(13), using 17 different radioactive amino acids, found a greater synthesis of nuclear protein in tumor tissue than in any other tissue studied. From these facts it appears that one of the major metabolic activities of tumor tissue was the production of nuclear proteins.

Summary. The specific activities of total liver protein and of the proteins of mitochondria, microsomes, and cell sap were higher in the protein-depleted rats than in the controls. Specific activities of these proteins in tumor-bearing rats, on the other hand, decreased below those in control animals. As the specific activity of the liver proteins of the tumor-bearing animals decreased, there was an increase in serum protein activity which then decreased as the tumor tissue continued to gain in isotopic activity. The data suggested that the protein-depleted animals had a decreased rate of synthesis of liver nuclear and serum albumin proteins. The data also indicated a rapid synthesis of nuclear protein in the tumors of tumor-bearing animals.

The author wishes to express appreciation to Miss Zina Sapankevych and Mr. Albert Schnerring who assisted in various phases of this study.

1. Garrow, J. S., *J. Clin. Invest.*, 1956, v38, 1241.

2. ———, Amino Acids Malnutrition, Rutgers University Press, New Brunswick, N. J., 1957.
3. Waterlow, J., *Nature,* 1959, v184, 1875.
4. Allison, J. B., Wannemacher, R. W., Jr., Russell, T., McCoy, J. R., *Cancer Res.,* 1958, v18, 398.
5. Allison, J. B., Wannemacher, R. W., Jr., Hilf, R., Migliarese, J. F., Crossley, M. L., *J. Nutrition,* 1954, v54, 593.
6. Zamecnik, P. R., Loftfield, B. L., Stephenson, M. L., Steele, J. N., *Cancer Res.,* 1951, v11, 592.
7. Carruthers, C., Woernly, D. L., Baumler, A., Davis, B., *ibid.,* 1959, v19, 59.
8. Hogeboom, G. H., Schneider, W. C., Stulich, M. J., *J. Biol. Chem.,* 1952, v196, 111.
9. Mider, G. B., *Cancer Res.,* 1951, v11, 821.
10. Henderson, L. M., Schurr, P. E., Elvehjem, C. A., *J. Biol. Chem.,* 1948, v177, 815.
11. Thompson, H. T., Schurr, P. E., Henderson, L. N., Elvehjem, C. A., *ibid.,* 1950, v182, 47.
12. Henderson, J. F., Le Page, G. A., *Cancer Res.,* 1959, v19, 749.
13. Busch, H., Davis, J. R., Honig, D. R., Anderson, D. C., Nair, D. V., Nyhan, W., *ibid.,* 1959, v19, 1030.

Received February 14, 1961. P.S.E.B.M., 1961, v107.

Connective Tissue III. Dermal Chemical Response to Age.* (26602

J. C. HOUCK, L. DE ANGELO AND R. A. JACOB
Biochemical Research Laboratory, Children's Hospital Research Foundation, and Department of Biochemistry, Georgetown Medical School, Washington, D.C.

Several investigators have shown that the collagen concentration of connective tissue increases with age(1-5) while changes in the hexosamine content of skin have been reported to decrease(2), remain constant(3) and even increase(1) with age. Kao and McGavack(6) have indicated that soluble dermal protein concentration decreased with age while there was a significant increase in the scleroprotein content of the tissue.

We have studied the effect of age upon the chemical anatomy of rat skin as revealed using a sequential extraction technic for dissection of skin into ground substance, neutral and citrate soluble collagens and insoluble collagen and scleroprotein(7). These results are described below.

Material and methods. Six groups of 12 male Sprague-Dawley rats weighing an average of 100, 150, 200, 270, 350 and 500 g each were sacrificed by etherization and exsanguination. About 2 g of abdominal skin were collected from each animal, shaven and dissected clean of adhering fat, fascia and muscle. Tissue from each animal was minced, and aliquots removed. The remaining minced skins were pooled into 2 collections from 6 animals each. Five g aliquots from each tissue pool were extracted sequentially in the cold with 0.15M NaCl, 0.50M NaCl, and 0.50M citrate buffer, pH 3.6 as has been described previously(7).

These extracts, as well as samples of skin from each animal separately (0.5 g) were hydrolysed in 4N HCl for 8 hours, and analyzed in triplicate for their hexosamine(8), hydroxyproline(9) and nitrogen(10) content. The results were expressed in μmoles per mmoles of dermal nitrogen to minimize variations in skin water and fat content. Subtraction of the sum of the soluble components from total dermal concentration permits the calculation of the insoluble dermal content of these tissues. These results were expressed as per cent of total hexosamine, collagen and noncollagenous protein which was insoluble.

Insoluble non-collagenous protein was calculated from the fact that one mg of collagen contains about one μmole of hydroxyproline and 13.3 μmoles of nitrogen(11). Therefore, the contribution of collagen nitrogen to total dermal nitrogen may be determined. The amount of non-collagenous nitrogen solubilized, when subtracted from total non-collagenous nitrogen, permits the amount of insoluble non-collagenous protein to be calcu-

*Supported in part by grant from Nat. Inst. of Arthritis and Metabol. Dis., P.H.S.

TABLE I. Effect of Age (in Terms of Body Weight) upon Chemical Construction of Rat Skin.

Analysis	Animal wt (g) 100	150	200	270	350	500
Total:						
Nitrogen*	3.75 ± .2	4.35 ± .3	4.70 ± .3	4.90 ± .3	4.15 ± .3	3.85 ± .2
Hexosamine†	2.50 ± .12	2.55 ± .12	2.17 ± .10	1.98 ± .10	1.45 ± .08	1.32 ± .07
Hydroxyproline†	28.8 ±1.4	35.4 ±1.8	38.5 ±1.9	49.0 ±2.4	53.0 ±2.7	47.5 ±2.4
.15M NaCl extract:						
Hexosamine†	1.69 ± .08	1.84 ± .09	1.51 ± .07	.93 ± .05	.43 ± .02	.36 ± .02
Hydroxyproline†	1.18 ± .06	1.91 ± .09	1.43 ± .07	1.04 ± .05	.70 ± .03	.57 ± .03
.50M NaCl extract:						
Hexosamine†	.83 ± .04	.73 ± .03	.66 ± .03	.39 ± .01	.39 ± .01	.34 ± .01
Hydroxyproline†	2.94 ± .15	3.45 ± .18	2.55 ± .13	4.00 ± .2	2.53 ± .13	1.17 ± .06
.50M citrate buffer:						
Hexosamine†	0	0	0	0	0	0
Hydroxyproline†	2.19 ± .10	2.60 ± .13	1.62 ± .08	1.63 ± .08	3.38 ± .16	3.38 ± .17
Insoluble hexosamine‡	0	0	0	35	44	50
Insoluble collagen§	65	71	82	85	87	95
Insoluble non-collagenous protein‖	50	35	22	2	21	46

* mmoles/g fresh skin. † μmoles/mmole whole skin nitrogen. ‡ % of total hexosamine. § % of total collagen. ‖ % of total non-collagenous protein.

lated from these results. The analytical results in terms of the mean of triplicate analyses of duplicate samples and their standard deviations are shown below. Duplicate extracts did not differ by more than 5%, and any means which differed by *more* than 10% were statistically significant ($p < .05$) for all the data presented below.

The 0.15M saline extract removed quantitatively the ground substance, while the 0.50M saline removed neutral soluble collagen(7). Citrate buffer effected the solubilization of the procollagen components of the skin(7).

Experimental and results: The analyses of whole skin and the various extracts are presented in Table I along with the calculations of percent of total hexosamine, collagen and non-collagenous protein which was insoluble. These results indicate that dermal nitrogen concentration increased with age until 270 g body weight was reached. Further increases in age (or weight) resulted in significant decreases in dermal nitrogen. Dermal hexosamine declined significantly with age, while hydroxyproline (or collagen) increased until an age equivalent to 350 g body weight was reached. Further aging then did not increase dermal collagen content.

Ground substance (0.15M saline soluble hexosamine) increased with age until 150 g body weight had been reached; further aging resulted in a significant decrease in ground substance. Neutral collagen bound hexosamine decreased with age, while no significant concentrations of hexosamine were found in the citrate buffer extracts.

Isotonic saline soluble hydroxyproline increased initially with age up to 150 g in body weight, then declined with further increases in body weight. Both soluble collagens changed in an apparently unmeaningful fashion with increasing animal weight and age.

No insoluble hexosamine was found until the animals reached a weight of 270 g. The percent of insoluble collagen increased continuously with age, while percent of non-collagenous scleroprotein decreased with age until 270 g body weight was reached; further aging results in increasing concentrations of dermal, insoluble, non-collagenous protein.

Summary and conclusions. Although the ground substance of rat skin appeared to decrease with age, dermal insoluble collagen content increased with age. In the face of decreasing concentrations of both total dermal hexosamine and ground substance, there was an abrupt appearance of insoluble hexosamine with age in excess of 200 g body

weight. Insoluble non-collagenous protein decreased with increasing body weight until 270 g. With further increases in age, dermal scleroprotein increased until the percent of total non-collagenous protein which was insoluble was essentially the same as that found in 100g rats. The maximum concentration of ground substance, total collagen and scleroprotein were found in the skin of rats weighing 150, 350 and 100g respectively. Maximum concentrations of insoluble hexosamine and insoluble collagen were found in the skin of rats weighing 500 and 270g respectively. The differing effects of aging upon ground substance, insoluble collagen and scleroproteins indicate that different mechanisms, possibly hormonal, were involved in controlling the concentration of each component, and suggest that these materials may be metabolically independent of each other.

1. Sobel, H., Marmorsten, J., *J. Gerontol.*, 1956, v11, 2.
2. Smits, G., *Biochim. Biophys. Acta.*, 1957, v25, 542.
3. Houck, J. C., Jacob, R. A., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 604.
4. Briscoe, A. M., Loring, W., McClement, J., *ibid.*, 1959, v102, 71.
5. Kao, K. T., Hilker, D. M., McGavack, T. H., *ibid.*, 1960, v104, 359.
6. Kao, K. T., McGavack, T. H., *ibid.*, 1959, v101, 153.
7. Houck, J. C., Jacob, R. A., *ibid.*, 1960, v105, 324.
8. Blix, G., *Acta Chem. Scand.*, 1948, v82, 467.
9. Martin, C. J., Axelrod, A., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 461.
10. Houck, J. C., Jacob, R. A., Maengwyn-Davies, G. D., *J. Clin. Invest.*, 1960, v39, 1758.
11. Neuman, R. E., Logan, M. A., *J. Biol. Chem.*, 1950, v186, 549.

Received February 24, 1961. P.S.E.B.M., 1961, v107.

Melanophore-Dispersing Activity of Reserpine in Rana Frogs.* (26603)

N. KHAZAN AND F. G. SULMAN
Department of Applied Pharmacology, Hebrew University—Hadassah Medical School and School of Pharmacy, Jerusalem, Israel.

The effect of reserpine on the endocrine system in mammals and man has been extensively studied(1-10). We wish to report here on the stimulating effect of reserpine on melanophore dispersion in *Rana pipiens* frogs.

Materials and methods. Fifty adult male and female green-adapted Rana frogs, weighing 30-60 g each, were used. Reserpine stock solution was prepared according to the method described in Martindale's Extra Pharmacopoea, 1959, and diluted to the required concentration. *Exp. 1*: Pituitary homogenates of Rana frogs were injected into 5 Rana frogs to establish the course and extent of the melanophore-dispersing activity of normal frog pituitaries. *Exp. 2*: Reserpine was added *in vitro* to Rana frog skins preserved in Ringer solution to determine whether reserpine has a direct effect on frog-skin melanophores. *Exp. 3*: Single doses of 0.05-0.25 mg reserpine were subcutaneously injected into the dorsal lymph sac of 10 frogs, while 10 other frogs were injected with the solvent alone. Each frog was kept in a separate vessel and watched for 4 weeks.

The degree of darkening was evaluated quantitatively by determining the melanophore index (M.I.) according to Hogben's melanophore scale method(11-14). The M.I. of the middle hind webs of the frogs was determined microscopically.

Results. Exp. 1: Injection of Rana frog pituitary homogenates into normal Rana frogs resulted in darkening of the skin within 6-12 hours. This effect passed within 48 hours. *Exp. 2*: No effect was observed when reserpine was added to Ringer solution-pre-

* This paper has been aided by a generous grant from Keren Nechuth, administered by Dr. S. Kott.

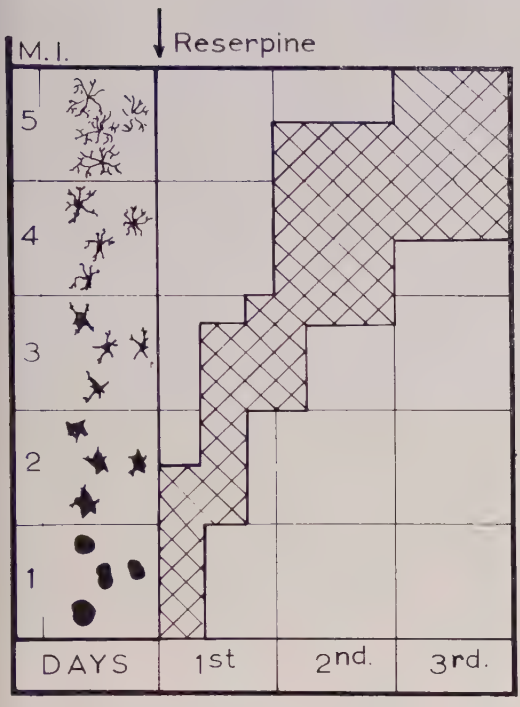


FIG. 1. Melanophore expansion in the large hind web of a Rana frog 3, 6, 12 hours and 2-3 days after injection, respectively. Melanophore index readings (M.I.) according to Landgrebe and Waring(14).

served Rana frog skins. *Exp. 3*: Within 3 hours of injection, the reserpine-treated frogs assumed a darker coloration and became almost black within 24-48 hours, 30% of them retaining their darker skin color for more than 3 weeks. The controls showed no discoloration.

Microscopic examination showed that within a few hours after injection of reserpine, the M.I. rose from the usual 1-2 to a maximum of 5 on the 2nd-3rd day (Fig. 1, 2, 3). Severe ptosis appeared within 1-2 days in all the treated frogs and lasted for 2-3 weeks.

Discussion. In mammals, reserpine was reported to interfere with the normal functioning of the anterior and posterior lobes of the pituitary, which effect was ascribed to its depressing action on the hypothalamus.

Reserpine in adequate doses caused suppression of anterior pituitary FSH and LH secretion(1,3,9), of TSH secretion(6,7) and of the exophthalmic factor(8). Elevation of prolactin secretion(4,5,9) and transient elevation of ACTH secretion followed by decline were also reported(10). The possibility of a stimulating reserpine effect on the pituitary posterior lobe function through release of ADH was also suggested(1) and confirmed by us. Our present experiments revealed an additional effect of reserpine on the posterior lobe of the pituitary, shown by increased endogenous secretion of MSH. Thus the following picture emerges: reserpine suppresses FSH, LH, TSH and ACTH, but increases LTH, ADH and MSH. This is in conformity with our hypothesis for the mechanism of the endocrine effects obtained with phenothiazine derivatives(15) which probably also holds true for reserpine(9). Following suppression of the hypothalamus, a secondary impairment of pituitary and peripheral glands occurs. With regard to part of the pituitary tropins (FSH, LH,

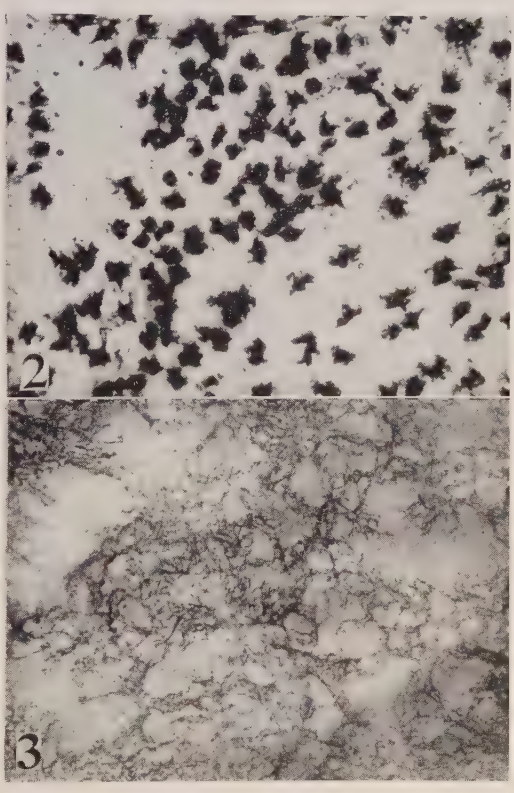


FIG. 2. Microscopic appearance of melanophores in the large hind web of a non-treated Rana frog; M.I.: 1-2 (× 100).

FIG. 3. Microscopic appearance of melanophores in the large hind web of Rana frog 3 days after s.c. injection of 0.1 mg reserpine; M.I.: 5 (× 100).

TSH, ACTH) this suppression becomes balanced according to the push and pull principle, which applies readily to the tropins producing secondary peripheral hormones (balanced tropins). With regard to those pituitary hormones which are not held in abeyance by the feed-back mechanism of their secondary peripheral hormones (LTH, ADH and MSH), an overshoot is to be expected. This would classify LTH, ADH and MSH as unbalanced tropins.

Our hypothesis has been confirmed recently by Scott and Nading(16). They reported that phenothiazine tranquilizers caused release of MSH by counteracting hypothalamic inhibition.

With regard to our experiments it is relevant that the melanophore effect was obtained only in Rana frogs and Bufo. Hyla frogs did not react to reserpine with darkening of the skin. This would indicate that the tree frog has a more stable regulation of MSH secretion than Rana. Hyla melanophores have indeed been considered to react more specifically to MSH and ACTH assays than those of Rana or Bufo(17,18).

Summary. Reserpine injected into the dorsal lymph sac of Rana frogs at dose levels of 0.05-0.25 mg was shown to provoke melanophore-dispersion in the skin. The melanophore index, M.I., in the hind webs of the frog rose from 1 to 5 within 24-72 hours after reserpine injection. Reserpine did not cause any change of skin color *in vitro*. It is suggested that this effect is due to intensified endogenous MSH secretion.

Our thanks are due Mr. E. Zawojski for devoted technical assistance.

1. Gaunt, R., Renzi, A. A., Antanchak, N., Miller, G. J., Gilman, M., *Ann. N. Y. Acad. Sci.,* 1954, v59, 22.

2. Tuchman-Duplésis, H., Mercier-Parot, L., *Compt. Rend.,* 1956, v243, 410.

3. DeFeo, W. J., Reynolds, S. R. M., *Science,* 1956, v124, 726.

4. Meites, J., Nicoll, C. S., Talwalker, P. K., PROC. SOC. EXP. BIOL. AND MED., 1959, v101, 563.

5. Robinson, B., *Med. J. Australia,* 1957, v2, 239.

6. Mayer, S. W., Kelly, F. H., Mortor, M. E., *J. Pharmacol. and Exp. Therap.,* 1956, v117, 197.

7. Bierwagen, M. E., Smith, D. L., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 108.

8. Assael, M., Gabai, F., Winnik, H. Z., Khazan, N., Sulman, F. G., *Lancet,* 1960, v1, 499.

9. Khazan, N., Sulman, F. G., Winnik, H. Z., PROC. SOC. EXP. BIOL. AND MED., 1960, v105, 201.

10. ———, *ibid.,* 1961, v106, 579.

11. Hogben, L. T., *Quart. J. Exp. Physiol.,* 1923, v13, 177.

12. Hogben, L. T., Gordon, C., *J. Exp. Biol.,* 1930, v7, 288.

13. Hogben, L. T., Slome, D., *Proc. Roy. Soc.,* 1931, B108, 10.

14. Landgrebe, F. W., Waring, H., *Quart. J. Exp. Physiol.,* 1944, v33 1.

15. Sulman, F. G., *Arch. int. pharmacodyn.,* 1959, v118, 298.

16. Scott, G. T., Nading, L. K., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 88.

17. Hochman, A., Ratzkowski, E., Sulman, F. G., *Acta Endocrin.,* 1954, v15, 389.

18. Lock, J. A., *Lancet,* 1952, v2, 147.

Received March 3, 1961. P.S.E.B.M., 1961, v107.

Distribution and Particle Size of Type A Botulinum Toxin in Body Fluids of Intravenously Injected Rabbits.* (26604)

G. J. HILDEBRAND, CARL LAMANNA, AND ROBERT J. HECKLY
Naval Biological Laboratory, School of Public Health, University of California, Berkeley

The present study reports distribution of type A botulinum toxin in various body fluids after intravenous administration of the toxin to rabbits. The sedimentation coefficient of the toxin in plasma and lymph was determined by partition cell ultracentrifuga-

* This work was sponsored by the Office of Naval Research and Bureau of Medicine and Surgery, U. S. Navy, under a contract between Office of Naval Research and Regents of University of California.

tion. These observations were undertaken to investigate whether or not exposure of the toxin to the *in vivo* environment might result in the appearance of toxophoric particles smaller in size than can be attributed to a protein molecule. Ingested toxin which appears in the lymph draining the intestinal tract has the dimensions of a protein(1).

Materials and methods. Concentration of toxin in specimens was expressed in LD_{50} values calculated by the method of Reed and Muench, employing white mice for the assay as previously described(1,2). Sedimentation coefficients (S_{20w}) of toxin in fluid specimens were calculated from measurements of the distribution of toxin between upper and lower portions of partition cells after ultracentrifugation as outlined by Heckly *et al.* (1). Crystalline type A toxin dissolved in 0.05M phosphate-0.2% gelatin buffer was injected into marginal ear veins of New Zealand white male and female rabbits weighing between 2 to 5 kg. This toxin had S_{20w} values of 12-21 when measured by the partition cell centrifugation method.

Sampling of body fluids. Pentobarbital –Na (30-60 mg) was injected intravenously when an anaesthetic was required.

Blood was taken from the right ventricle at termination of an experiment. Consecutive samples of blood were taken from the cannulated jugular or femoral vein through a polyethylene tube flushed with saline both before and after each sample was taken. Heparin was added to the collected blood. Lymph was taken by continuous sampling from cannulated left thoracic duct, and collected under ice to avoid clotting without addition of anticoagulant. Fetal blood was taken from 3- to 4-week pregnant rabbits at the time of maternal respiratory failure. The fetus was exposed, the thorax opened and blood aspirated from the right ventricle. Blood from fetuses of a litter was pooled. Spinal fluid was obtained by suboccipital puncture of the cisterna cerebello-medullaris. Ocular fluid was taken through a No. 26 needle inserted in the lateral rim of the cornea and directed towards the middle of the anterior eye chamber. For continuous sampling, the needle was left in place and the ocular fluid allowed to drip into a vial through a small polyethylene tube attached to the needle. Bile was taken from the gall bladder at termination of an experiment. In some cases the common bile duct was cannulated and the bile collected continuously under ice. Urine was taken from the urinary bladder at termination of an experiment or the bladder was catheterized and the urine sampled continuously under ice. Specimens of body fluids showing macroscopic evidence of blood contamination were discarded.

Results. The particle size of botulinum toxin is a function of its degree of association with a separable hemagglutinating protein(2). Since dissociation of the hemagglutinin from the toxic entity might occur *in vivo,* it was important to establish the sedimentation coefficient of toxin freed from the hemagglutinin. Consequently, hemagglutinin-free toxin was prepared by exposing crystalline toxin dissolved in an isotonic sodium chloride-phosphate buffer, pH 7.3, at a maximum temperature of 10°C to successive batches of chicken erythrocytes. Under these conditions selective adsorption to the red cells reduced the hemagglutinin titer of the toxin solution to zero. While the method results in losses of toxin, the toxin remaining in solution is free of hemagglutinin after centrifuging out the red cells. This hemagglutinin-free toxin was found to have S_{20w} values in the range of 3.7 to 8.4.

Since heparin was employed at times *in vivo* and *in vitro,* it was necessary to learn whether or not this might affect the assay for toxin. Titrating the blood and plasma of 2 heparinized rabbits given toxin resulted in 100% recovery of the expected toxicity values. Thus, *in vivo* use of heparin does not appear to affect the assay for toxicity.

In vitro heparin can precipitate toxin dissolved in salt solutions. The precipitation, however, can be prevented or reversed by addition of serum albumin. Ultracentrifugation revealed that the sedimentation coefficient of toxin in plasma and serum under the conditions of the present investigation was unaffected by the presence of heparin or citrate. On the other hand, when these anticoagulants were added to a salt solution of

TABLE I. Distribution of Toxin between Plasma and Whole Blood in Intravenously Injected Rabbits.

Dose (mouse LD_{50}/kg)	Sample time after inj., min.	Mouse LD_{50}/ml*	
		Whole blood	Plasma
200,000	120	3,200	6,000
400,000	120	6,400	12,000
1,000,000	120	14,000	28,000

* Toxic blood removed from rabbit, and blood titrated. A sample specimen was centrifuged at 10°C for recovery and titration of plasma. Since red cells occupy about 40% volume of blood, the approximate 2-fold greater toxicity of plasma than whole blood argues in favor of the complete presence of the toxin in the liquid phase of blood.

toxin, increased values for the sedimentation coefficient of toxin were found. It is therefore felt that the sedimentation coefficients of toxin in plasma and lymph reported here are independent of the presence of added anticoagulant.

Since toxin was injected directly into the blood stream, the possibility was explored that toxin might be distributed between erythrocytes and plasma. It can be concluded from the data in Table I that all of the toxin remains in the liquid phase.

Toxin in plasma and lymph. The toxicities of plasma and lymph were determined at various time intervals after injection of 4 rabbits with 2×10^6 mouse LD_{50}/kg. Toxin could be demonstrated in lymph within 25 minutes, though the highest concentrations required longer periods for their appearance (Table II). The toxicity of lymph did not exceed between 1/6 to 1/12th part the toxicity of plasma. Thus toxin appears to escape slowly from blood to the lymph collectable from the thoracic duct. The disproportion between the toxicity of plasma and lymph could be solely a reflection of a lack of time to reach a true plateau value. To answer this question, it would be necessary to repeat this experiment employing lower levels of toxin, to permit survival of groups of animals for significantly longer periods of time.

Determinations of sedimentation coefficients of the toxin are recorded in Table II. The size of the toxic material in lymph and plasma cannot be said to differ. The variations in sedimentation coefficients are within the limits expected from the large experimental errors inherent in the procedures employed. What is worth noting is that all the values are in a range expected for proteins. The size of the toxin after intravenous injection is significantly less than that of crystal-

TABLE II. Toxicity and Sedimentation Values of Toxin in Plasma and Lymph of Rabbits Intravenously Injected with 2,000,000 Intraperitoneal Mouse LD_{50} per kg of Subject Rabbit.

Rabbit	Sampling time post-inj., min.	Plasma		Lymph		Death (D) or termination (T) time, min.
		Toxicity, mouse LD_{50}	*S_{20w}	Toxicity, mouse LD_{50}	*S_{20w}	
A	5	113,000	13.9	—	—	
	24	113,000	9.1	700	—	
	40	74,800	12.2	800	—	
	55	64,000	12.5	5000	—	55 D
B	4	50,000*	4.2	—	—	
	20	61,000*	10.0	236*	7.7	
	40	43,000*	5.9	2300*	10.3	
	90	43,000*	6.4	3100*	11.4	
	100	—	—	2600*	6.6	100 T
C	5	80,000	4.0	—	—	
	20	—	—	57	—	
	40	77,000*	7.3	7800	14.7	
	70	57,000	9.6	9100	14.2	
	77	—	—	9600	7.4	77 D
D	1	80,000	11.2	—	—	
	25	57,000	8.2	<16	—	
	110	64,000	12.6	4000	9.0	
	130	—	—	3800	7.0	130 T

* Toxicity values are based on a calculation from toxicities of top and bottom specimens of the ultracentrifuge partition cell by the method recorded in Heckly *et al.*(1).

TABLE III. Test for Presence of Toxin in Pools of Fetal Blood of Litters from Rabbits at Time of Maternal Respiratory Failure after Intravenous Injection with Type A Crystalline Botulinum Toxin.

Rabbit	Dose (mouse LD_{50}/kg)	Toxicity of blood*: Maternal	Toxicity of blood*: Fetal	Respiratory failure time, min.
1	400,000	6,000	0	70
2	1,000,000	16,000	0	87
3	1,000,000	12,000	0	114

* Expressed as mouse LD_{50}/ml of blood. With fetal blood the method employed would permit detection of 2 or more mouse LD_{50}/ml.

line toxin. This could be due to partial dissociation of hemagglutinin units from the toxic entity. The S_{20w} values obtained are too large to permit the conclusion that the toxin becomes completely free of hemagglutinating material. The measurements are similar to those found for toxin appearing in lymph taken from rats given crystalline toxin intraduodenally (1).

Toxin in fetal blood. Toxin was absent in 3 pools of fetal blood taken at the time of respiratory failure of the poisoned parents. The toxicity of maternal blood was about 10,000 mouse LD_{50}/ml (Table III). There appears to be no rapid transfer of toxin from maternal to fetal blood.

Toxin in ocular fluid. Ocular fluid was taken from rabbits at periods from 30 to 120 minutes after intravenous doses of toxin were given in the range from 200,000 to 2,000,000 mouse LD_{50}/kg. Upon initial puncture of the anterior eye chamber ocular fluid was collected with no detectable toxicity for the mouse in 20 out of 33 experiments. The technic of titration employed would give such a negative result if less than 10 mouse LD_{50} doses were present per ml of ocular fluid, and would represent values of toxicity for the ocular fluid of less than 1/400 to 1/3600 part of the toxicity present per ml of plasma of the rabbits. In the remaining 13 experiments detectable toxicity was present, the highest value recorded being about 5% of the toxin in one ml of plasma. Contamination of the ocular fluid with blood can be excluded as a cause of the presence of toxin in these positive instances. The toxicity values obtained could be correlated neither with amount of the injected toxin nor interval of time between injection of the toxin and sampling of ocular fluid. It would appear that normally a barrier exists to the passage of toxin from blood to ocular fluid but that this barrier varies with individual animals. Inducing inflammation of the eye can cause the appearance of some toxin in the intraocular fluid (Table IV).

In 14 experiments, after the initial withdrawal of ocular fluid, sampling was permitted to go on continuously. In these instances the ocular fluid was drained as fast as it was formed, which would permit an increase in the difference between intracapillary and intraocular pressures. Under these circumstances of reduced intraocular pressure the toxicity of the ocular fluid rose substantially, in some cases to values approaching that for plasma. Since rupture of blood vessels could be excluded as the cause of this rise in toxicity, the presence of toxin in the ocular fluid can be attributed to filtration processes. Table IV gives data in support of this conclusion. That filtration is the prob-

TABLE IV. Accumulation of Toxin in Ocular Fluid under Conditions of Normal and Low Intraocular Pressure.

Rabbit	Time of sampling postinjection, min.	Toxicity (mouse LD_{50}/ml): Blood	Ocular fluid: Normal	Ocular fluid: Low pressure*
A	30	16,000	0 (one eye) 20 (other eye)†	
B	60	6,000	0 (one eye)‡	8,000§
	90	6,000	0 (other eye)	
C	60	8,000	0 (one eye)	6,000
	90	8,000	0 (other eye)	
D	30		0 (one eye)	8,000
	120	6,000		

* Low pressure achieved by continuous tapping of ocular fluid for time interval indicated.

† This eye was artificially inflamed by instilling allyl-isothiocyanate into conjunctival sac.

‡ One eye sampled and ocular fluid found to be non-toxic, then allowed to drain continuously to prevent intraocular pressure from building up to normal, and ocular fluid retested for toxicity. The other intact eye was then sampled at the same time.

§ The higher toxicity in ocular fluid than in blood is due to the fact that toxin is limited to plasma. Blood toxin values should be approximately doubled for plasma toxin values (Table I).

able mechanism for the appearance of toxin in ocular fluid is also supported by an experiment in which, after removal of ocular fluid, saline was used to replace the fluid and thus prevent anything other than a transient reduction in intraocular pressure. In this situation the ocular fluid remained non-toxic after retapping. Maintaining the intraocular pressure acts against escape of toxin from the capillaries of the eye, as would be expected if toxin enters the intraocular fluid by a filtration mechanism.

Toxin in urine and bile. Toxin mixed *in vitro* with urine and bile was inactivated at body temperature, particularly in the case of bile. The quantitative aspects of these inactivations have not been worked out, but the phenomenon shows that finding an absence of botulinum toxin in urine or bile which has remained for a time in the fluids' reservoirs might reflect toxin inactivation and not a true inability of the toxin to make its way into these body fluids. This might explain Legroux and Levaditi's(3) findings of toxin in blood of injected rabbits with the occasional failure to find toxin in urine taken directly from the bladder. Therefore, a series of experiments were performed by direct cannulization of the urinary bladder and the common bile ducts. The specimens were collected under ice for one hour and then immediately injected intraperitoneally into mice. The specificity of deaths was authenticated by including among the injected mice individuals which had been passively immunized with specific antitoxin.

To exclude the possibility that circulatory failure after large intravenous injections of toxin would interfere with excretory functions of the liver and the kidney, mean arterial pressure was recorded in the femoral artery. During the first hour after intravenous injection of 10,000 to 1,000,000 mouse LD_{50}/kg of toxin, the blood pressure of the rabbits did not fall below 100 mm Hg. Only during the terminal stages of asphyxia following respiratory failure did arterial pressure change significantly.

The urine collected within an hour from 6 rabbits receiving intravenous injections of 1,000,000 mouse LD_{50}/kg contained 30 to 230 mouse LD_{50}/ml. In 3 experiments bile was toxic to the extent of 100 to 140 mouse LD_{50}/ml. It is evident that toxin can appear in urine and bile. In relation to the dose received, the amount of toxin eliminated by these routes is too small to signify a therapeutically significant loss of toxin. Since it is well known that small quantities of protein occur in the excretions of the livers and kidneys of normal animals, the toxin appearing in urine and bile cannot be considered unique examples of passage of protein through these organs.

Cerebrospinal fluid. From 10 rabbits receiving 200,000 to 1,000,000 mouse LD_{50}/kg, specimens of cerebrospinal fluid were negative for toxicity in 9 cases. Four of the negative specimens were drawn 30 minutes postinjection, 4 were drawn 90 minutes postinjection, and 1 drawn 120 minutes postinjection. The one specimen showing toxicity (10 mouse LD_{50}/ml) was taken at time of death (100 minutes) from a rabbit having received 200,000 mouse LD_{50}/kg with a blood level of 3,000 mouse LD_{50}/ml. The experience cited does not point to capacity of toxin to pass readily into the cerebrospinal fluid, at least during the time intervals of our experiments, and does not exclude the possibility for appearance of small quantities of toxin under conditions other than those employed. One might infer that in natural cases of food poisoning, where much lower levels of toxin will appear in blood than in these experiments, the presence of measurable amounts of toxin in cerebrospinal fluid will be quite doubtful. No toxin could be found in cerebrospinal fluid of a rabbit 10 hours after 11,000,000 mouse LD_{50} were instilled intraduodenally and a toxic blood level of 160 mouse LD_{50}/ml had been achieved.

Discussion. The distribution of intravenously injected type A toxin seems to parallel the distribution of protein in body fluids. Apart from plasma, only lymph appears to show a consistently high toxicity. The finding that increase of filtration pressure will lead to a greatly increased toxicity of the ocular fluid is compatible with the assumption that a toxic protein is passing by filtration from the blood stream into other body

fluids. Our data are in accord with the behavior of proteins in plasma and the knowledge that the glomerular membrane is relatively impermeable to protein in spite of a high filtration rate in the kidney.

The data suggest that in diagnosis of poisoning by botulism it is advisable to collect blood for toxicity tests. It should be pointed out, however, that in human cases brought to autopsy toxin has been detected in extracts of liver when blood specimens have been negative(4).

Summary. In rabbits which were given type A botulinum toxin intravenously, and which died rapidly of acute poisoning the toxin was found in lymph and plasma with no tendency to diffuse into erythrocytes. Ratio of toxin in lymph to plasma ranged between 1:6 to 1:12. Sedimentation coefficients of the toxin in these body fluids were similar and had the dimensions of a protein. The particle size was decreased in relation to crystalline toxin, was similar to toxin in lymph from rats given toxin intraduodenally, but was not sufficiently smaller to warrant the assumption of complete dissociation of toxin from the hemagglutinin present in crystalline toxin.

Toxin was absent from fetal blood, but appeared in small quantities in urine and bile. Cerebrospinal fluid was free of toxin except for a small amount at death. In the normal eye none or only a small quantity of toxin appears in the intraocular fluid. By experimental reduction of intraocular pressure toxin in intraocular fluid tends to approach concentrations present in plasma. Inflammation can lead to the occurrence of toxin in intraocular fluid. The findings are compatible with the hypothesis that the toxin *in vivo* has the dimensions of protein and passes tissue barriers by filtration mechanisms normally operative.

1. Heckly, R. J., Hildebrand, G. J., Lamanna, C., *J. Exp. Med.*, 1960, v111, 745.

2. Meyer, E. A., Lamanna, C., *J. Bact.*, 1959, v78, 175.

3. Legroux, R., Levaditi, J. D., *Compt. Rend. Soc. Biol.*, 1947, v141, 996.

4. Schneider, H. J., Fisk, R., *J. Am. Med. Assn.*, 1939, v113, 2229.

Received March 6, 1961. P.S.E.B.M., 1961, v107.

Amino Acid Composition of Mycobacterial Cell Wall.* (26605)

DELLA R. BELKNAP, MERRILL N. CAMIEN AND MAX S. DUNN
Chemical Laboratory, University of California, Los Angeles

Determinations of mycobacterial cell wall composition have been made semi-quantitatively for taxonomic purposes by Cummins and Harris(1), and quantitatively by Kotani, *et al.*(2), who also studied particulate and soluble fractions from the same mycobacterial cells. Glutamic acid, alanine, and α,α'-diaminopimelic acid were not determined quantitatively by the latter authors but were identified as cell wall components. Antigenic properties of mycobacterial cell wall have been examined by Ribi, *et al.*(3). The present report concerns the quantitative determination of amino acids in cell walls from 4 mycobacteria.

Materials and methods. Cells of *Mycobacterium tuberculosis,* strains† $H37R_a$, and

* Paper 137. Contents taken in part from Miss Belknap's Master's thesis. This work was supported by the Emma Wade Scott bequest to the Nat. Tuberculosis Assn. and by grants from Los Angeles County Tuberculosis and Health Assn., U.S.P.H.S., and Univ. of California. The authors are indebted to Dr. Seymour Froman and the Olive View Hospital, Olive View, Calif., for generous provision of facilities and cultures, to E. S. Bowler and F. W. Bishop for indispensable assistance with the electron microscopic studies, and to Audree V. Fowler for helpful suggestions and assistance.

† All cultures were supplied by Olive View Hospital through the courtesy of Dr. Seymour Froman. Strain numbers other than the familiar $H37R_a$ are those of the Olive View Hospital culture collection catalogue.

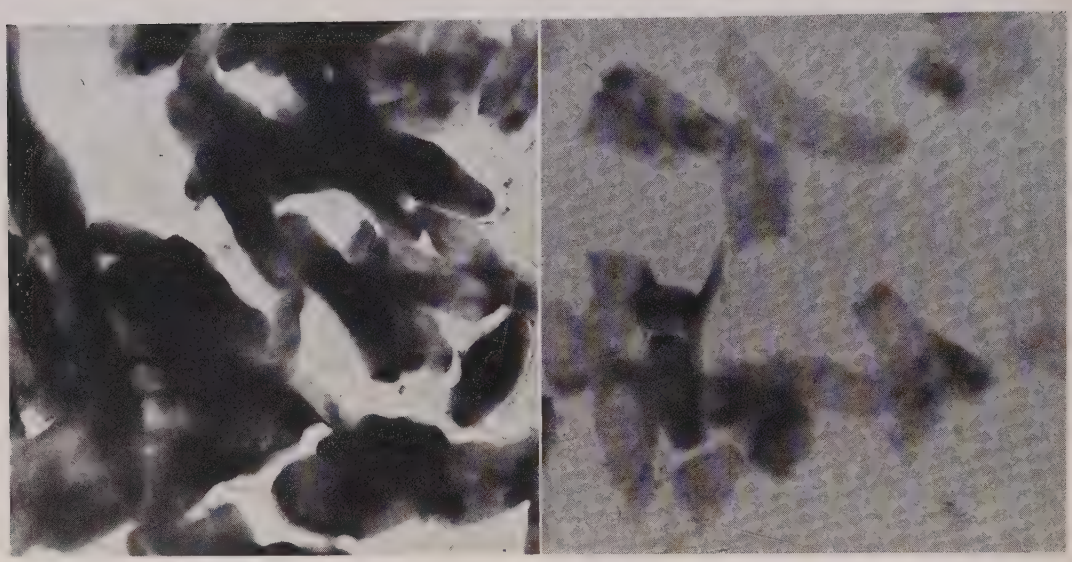


FIG. 1. Electron micrographs of unshadowed and unstained preparations derived from *Mycobacterium sp.*, 'Battey' strain F811. *Left*—partially defatted, air-dried cells which were subsequently subjected to 50 consecutive washings with distilled water (some cell contents are removed by this process). *Right*—cell wall material from the same organism.

F1136, *Mycobacterium ranae,* and the unclassified 'Battey' strain F811, were grown in Roux bottles on Sauton's medium at 37° until maximal pellicle formation had been achieved without onset of degeneration. The cultures of *M. tuberculosis* $H37R_a$ and *M. ranae* were harvested directly, but strains F811 and F1136 (pathogenic) were first sterilized by shaking them with equal volumes of acetone and allowing the mixtures to stand 24 hours. Each lot of cells was harvested by filtration on a Buchner funnel, washed with distilled water, and partially defatted by consecutive 24 hour extractions with acetone and diethyl ether. The air-dried products were stored at room temperature.

Two g portions of cells of each strain were suspended in 30 ml of 0.67 *M* phosphate buffer, pH 7.4, for fragmentation and separation by the method of Kotani, *et al.*(2). Extraneous cellular material was further eliminated by digestion with trypsin and pepsin as described by Cummins and Harris(1), and after final washings the apparently clean cell walls were dried by lyophilization. Absence of cell contents was demonstrated by electron micrography of unshadowed and unstained preparations (Fig. 1). In the case of one batch of cell walls, which will be referred to as "ranae 2," the lyophilized preparation was suspended in phosphate buffer and subjected to an additional separation by the method of Kotani, *et al.*(2).

The products were hydrolysed by heating them in 3 *N* hydrochloric acid in sealed glass tubes for 24 hours at 130°, and the hydrolysates were analysed by the quantitative column chromatographic method of Moore and Stein(4)‡. The chromatographic procedure was checked for precision by running most of the analyses in duplicate and for accuracy by use of recovery samples. The cell wall analyses were verified qualitatively by paper chromatography on Whatman No. 1 paper with the solvent systems, 95% ethanol-concentrated ammonium hydroxide - water 8:1:1, and methanol - 1.25 *N* hydrochloric acid - pyridine 8:2:1.

Results and discussion. Satisfactory pur-

‡ The resin employed was the Analytical Grade of Dowex 50W-x4, minus 400 mesh. Independent ninhydrin color standards prepared with alanine, glutamic acid, diaminopimelic acid and leucine, respectively, were employed in estimating these 4 amino acids in the chromatographically analysed samples. The remaining amino acids were estimated with respect to the leucine ninhydrin color standard, assuming essentially equivalent color yields.

TABLE I. Amino Acid Content of Mycobacterial Cell Wall Preparations.*

Amino acid	Cell wall source†				
	ranae	ranae 2	$H37R_a$	F1136	F811
Glutamic acid	31.8 (5.4, 5.5)	36.5 (5.4)	37.6 (6.8, 6.4)	41.6 (5.4, 5.5)	22.9 (3.2, 3.4)
Alanine	28.9 (3.0, 3.0)	33.5 (3.0)	22.6 (2.4, 2.4)	26.5 (2.1, 2.1)	31.5 (2.8, 2.7)
Diaminopimelic acid	13.6 (3.1, 3.0)	16.1 (3.1)	15.9 (3.6, 3.7)	17.8 (3.1, 3.0)	10.9 (2.1, 2.0)
Aspartic acid	5.8 (.9, .9)	6.7 (.9)	4.4 (.7, .7)	.0 (.0, .0)	11.5 (1.6, 1.4)
Serine	3.3 (.4, .4)	3.8 (.4)	4.8 (.7, .5)	.0 (.0, .0)	5.8 (.7, .5)
Threonine	3.2 (.4, .5)	3.3 (.4)	2.8 (.4, .4)	.0 (.0, .0)	6.0 (.7, .7)
Glycine	6.9 (.6, .6)	.0 (.0)	5.6 (.6, .4)	.0 (.0, .0)	11.5 (.9, .8)
Isoleucine	.0 (.0, .0)	.0 (.0)	6.4 (1.0, 1.0)	5.1 (.6, .6)	.0 (.0, .0)
Leucine	.0 (.0, .0)	.0 (.0)	.0 (.0, .0)	9.0 (1.1, 1.0)	.0 (.0, .0)
Valine	6.6 (.9, .9)	.0 (.0)	.0 (.0, .0)	.0 (.0, .0)	.0 (.0, .0)

* Avg values listed as moles/100 amino acid residues. Original weight % values given in parentheses. Unlisted amino acids enumerated in text could not be detected by either column or paper chromatography, although amounts corresponding to one-tenth the threonine level in $H37R_a$ (column 3) would have been readily measurable if present.

† See text for strain designations and distinction between "ranae" (column 1) and "ranae 2" (column 2).

ity of the cell wall preparations was indicated by their relative freedom from adhering cytoplasmic contents in electron micrographs (representative prints shown in Fig. 1)§ and by absence of chromatographically detectable amounts of arginine, cystine, histidine, lysine, methionine, phenylalanine, proline and tyrosine in their acid hydrolysates (amino acid analyses are given in Table I)‖. Reliability of the amino acid analyses was indicated by good agreement between duplicate determinations (Table I), quantitative recovery of each of the amino acids listed in Table I from known mixtures (typical recoveries ranged from 96 to 104%), and qualitative agreement between analyses by paper chromatography (2 solvent systems) and column chromatography. Essentially complete data from a typical elution analysis are shown graphically in Fig. 2.

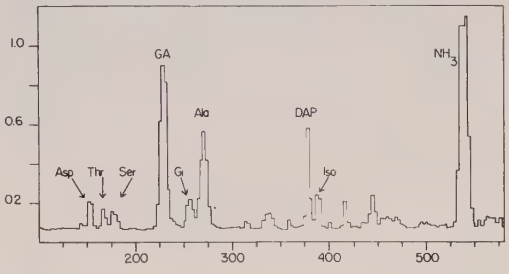


FIG. 2. Elution pattern of amino acids in hydrolysate of cell walls from *Mycobacterium tuberculosis* $H37R_a$. Labeled elution peaks correspond to aspartic acid, threonine, serine, glutamic acid, glycine, alanine, diaminopimelic acid, isoleucine, and ammonia, respectively. Unlabeled irregularities in the base line did not recur in replicate analyses and hence do not appear to have derived from the cell wall hydrolysate.

Glutamic acid, alanine and diaminopimelic acid were major cell wall components in each of the 4 mycobacteria (Table I), occurring in approximate molar ratios of 2:2:1, 2:3:1 and 5:3:2 in *M. ranae* (2 cell wall preparations), the unclassified strain F811, and *M. tuberculosis* (both strains), respectively. Aspartic acid, serine, threonine and glycine (Table I) were either major or minor cell wall components in 3 of the mycobacteria, but none of these 4 amino acids was detected in the preparation from *M. tuberculosis* F1136¶. Isoleucine and leucine were found

§ Most published electron micrographs of cell walls have been produced from metal-shadowed specimens and hence are not readily comparable with the unshadowed materials shown in Fig. 1. Shadowing was purposely avoided in the latter preparations since it seemed probable that the resulting electron-dense coating on the outer surfaces of the cell walls would tend to obscure residual cytoplasmic contents if such were present.

‖ Any of the amino acids listed as absent from the cell walls would have been readily measurable if present at one-tenth the level of threonine in $H37R_a$. (Table I). Even smaller amounts would have been detectable but not accurately measurable.

¶ The hydrolysate of this preparation was regrettably subjected to a decolorization with charcoal. It does not seem likely, however, that this treatment accounts for the apparent complete absence of any amino acid.

in cell walls only from the species *M. tuberculosis* (Table I), isoleucine being present in the material from both strains (leucine only from strain F1136). Valine was found in the cell walls only from *M. ranae*. Ammonia, which was incidentally determined in the chromatographic analyses, was present in all of the cell wall hydrolysates at molar levels approximating that in each case of glutamic acid, suggesting that the latter residue in the cell wall structure is normally amidated.

The analyses given in Table I, together with the semi-quantitative data reported earlier by Cummins and Harris(1), suggest that glutamic acid, alanine and diaminopimelic acid are major cell wall components in most or all mycobacteria, although it is evident (Table I) that the molar ratios of these 3 amino acids vary significantly in the cell walls of different mycobacterial species. Additional amino acids appear to be major cell wall components in certain mycobacteria. This is particularly noteworthy in the cell walls of strain F811 (Table I), which contain aspartic acid and glycine at the same molar level as diaminopimelic acid together with serine and threonine at approximately half this molar level. Similarly it may be seen (Table I) that the cell walls of *M. ranae* contain aspartic acid, glycine and valine, each at half the molar level of diaminopimelic acid and that the material from *M. tuberculosis* F1136 contains leucine at half the molar level of diaminopimelic acid. The remaining amino acids, where present (Table I), are generally at not less than one-fourth the molar level of diaminopimelic acid (the only exception is threonine at approximately one-eighth the diaminopimelic acid level in material from *M. tuberculosis* $H37R_a$) and hence may be considered probably significant.

The differences in composition between cell wall preparations "ranae" and "ranae 2," Table I, appear to be of considerable interest. Both glycine and valine are absent from "ranae 2," but are present in "ranae" at molar levels approximately half that of diaminopimelic acid. Possibly the glycine and valine observed in "ranae" were derived from contaminating material which was eliminated by further processing in "ranae 2," although this seems unlikely because the eliminated contaminating material would have to be constituted almost entirely of glycine and valine in order to account for these results. It is tentatively assumed, therefore, that glycine and valine are normally present in cell walls of *M. ranae* but are rendered sensitive to cleavage from the remaining structure by lyophilization, thus accounting for their absence from "ranae 2," which was subjected to an additional separation procedure after having been lyophilized. An apparently analogous solubilization of bacterial cell wall components following lyophilization has been reported by Brown (5).

It would be desirable, if possible, to find some feature in the cell wall composition of pathogenic strains of mycobacteria distinct from that of non-pathogenic strains, which might, therefore, relate cell wall structure to virulence in these organisms. The present data, although more extensive than previously available with regard to amino acid composition of mycobacterial cell walls, is evidently still too limited to reveal whether or not such a distinction exists.

Summary. The amino acid composition of cell walls from 4 strains of mycobacteria has been determined by quantitative column chromatography. Glutamic acid, alanine and diaminopimelic acid were major components in each of the cell wall preparations. Aspartic acid, serine, threonine, glycine, isoleucine, leucine and valine were present at probably significant levels in cell walls of one or more, but not all of the mycobacteria. Chromatographically detectable amounts of arginine, cystine, histidine, lysine, methionine, phenylalanine, proline and tyrosine were absent from all cell wall preparations.

1. Cummins, C., Harris, W., *J. Gen. Microbiol.*, 1956, v14, 583.

2. Kotani, S., Kitsura, T., Hirano, T., Tanaka, A., *Biken's J.*, 1959, v2, 129.

3. Ribi, E., Larson, C. L., List, R., Wicht, W., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 263.

4. Moore, S., Stein, W. H., *J. Biol. Chem.*, 1954, v211, 893.

5. Brown, A., *Biochim. Biophys. Acta*, 1958, v28, 445.

Received March 10, 1961. P.S.E.B.M., 1961, v107.

Hepatic Uptake and Intestinal Absorption of Co^{58}-Labelled 5,6-dimethylbenzimidazolylcobamide Coenzyme.* (26606)

Duk Ho Lee and George B. Jerzy Glass
Gastroenterology Research Laboratory, Department of Medicine, New York Medical College, New York City

The isolation of 5,6-dimethylbenzimidazolylcobamide coenzyme(1), (referred to here, for simplicity, as B_{12} coenzyme) has raised several questions regarding its turnover in the human body and its physiological significance in the metabolism of vit B_{12}. Although its role in the intermediate metabolism of amino acids has been established, very little has yet been published on the absorption, tissue distribution and urinary and fecal excretion of this substance(2-6). Twenty-four hours after intravenous injection of both labelled coenzyme and labelled cyanocobalamin to normal rats, more radioactivity was found in the kidneys than in the liver(5,6). Four to 5 days later, the reverse was true, *i.e.* more radioactivity was found in the liver than in the kidneys(5,6). This sequence is the opposite of that usually found, under similar conditions, after administration of cyanocobalamin(7,8). It was also noted that release of the parenterally administered coenzyme from the liver, through the bile, into the feces proceeds more slowly in rats than does that of cyanocobalamin(5). Hence the conclusion that the liver's affinity to coenzyme is greater than to cyanocobalamin(5, 6).

It was also demonstrated, by measurements of fecal radioactivity in the normal rat that there is much less absorption of orally administered coenzyme than cyanocobalamin at the same dosage(4-6). Comparative studies on humans have so far been few and fragmentary(2,3,5). The evidence available is scanty as to how much coenzyme, as compared with cyanocobalamin, is absorbed in the intestines and deposited in the liver of normals or patients with pernicious anemia. The present investigation was undertaken to answer some of these questions.

Material and method. For this study, we used 3 normal subjects and 3 patients with pernicious anemia in remission. The diagnosis of pernicious anemia was established by all available clinical and hematological criteria, including isotope assay of the intestinal absorption of labelled Vit. B_{12} and microbiological assay of the B_{12} serum levels. Co^{58}-labelled coenzyme of 0.616 $\mu c/\mu g$ specific activity and Co^{58}-labelled cyanocobalamin of 0.82 $\mu c/\mu g$ specific activity† were given orally to each subject at a dose of 2 μg after the radioactivity of the materials had been cut by physical decay by one-third to one-half of its initial potency. Each normal subject received (1) 2 μg of labelled cyanocobalamin and (2) 2 μg of labelled coenzyme at intervals of 15 to 20 days. At 2 to 2½ week intervals, each pernicious anemia patient received (1) 2 μg of labelled coenzyme; (2) 2 μg of labelled coenzyme together with 7.5 mg of hog intrinsic factor preparation # WES 727 (Lederle)‡; (3) 2 μg of labelled cyanocobalamin; (4) 2 μg of labelled cyanocobalamin together with 7.5 mg of the same intrinsic factor preparation. The sequence in which these 4 materials were given was changed from patient to patient.

The hepatic uptake of both labelled coenzyme and labelled cyanocobalamin was determined by the surface counting technic described earlier(9,10). Following oral administration of each of these materials, hepatic uptake of radioactivity was measured on 3 surface skin projections of the liver (anterior, antero-lateral and mid-lateral). In addition, 2 projections of the intestine (suprapubic and left lower quadrant) were counted as controls. The radioactivity over the left

* Supported by a research grant-in-aid from Squibb Inst. for Medical Research, New Brunswick, N. J.

† Both materials generously provided by Dr. C. L. Ravaris, Squibb Inst. for Medical Research, New Brunswick, N. J.

‡ Generously supplied by Dr. L. Ellenbogen of Lederle Labs. Division, Am. Cyanamid Co., Pearl River, N. Y.

TABLE I. Hepatic Uptake of Radioactivity Following Administration of Co^{58}-coenzyme and Co^{58}-cyanocobalamin.

	Name	Diagnosis	Cyanocobalamin, coenzyme B_{12}, administered	Hepatic uptake in cpm/μc Co^{58}	
				Cyanocobalamin	Coenzyme B_{12}
1.	Clar.	Normal	Alone	309	172
2.	Met.	"	"	371	457
3.	Mla.	"	"	—	232
4.	Sull.	Pernicious anemia	Alone	5	0
	"	" "	With IF	397	254
5.	Lea.		Alone	0	0
	"	" "	With IF	330	119
6.	Mea.	" "	Alone	0	0
			With IF	316	207

calf muscle was counted as body background. The counting areas were marked with indelible stain and adhesive tape to insure identical counting projections throughout the investigation. Each count was taken for at least 10 minutes, by means of a scintillation counter provided with a sodium iodide-thallium crystal ($\frac{3}{4}'' \times 1''$) and a gamma spectrometer, attached to a scaler, and were taken at the spectrometer's optimal energy setting for Co^{58}. The counting time was long enough to secure statistically significant counts(10). The hepatic and intestinal counts were averaged separately. The body background was deducted and the counts were corrected for physical decay of the material and for efficiency of the scaler with the use of a Co^{58} standard.

Results. The results are tabulated in Fig. 1 and 2 and in Table I. In normals, absorption of coenzyme B_{12} in the intestine and its deposition in the liver was, in one case, 23% higher, and, in another, 40% less, than that of cyanocobalamin (Fig. 1). In the pernicious anemia patients, when cyanocobalamin and coenzyme were given without an additional source of intrinsic factor (Table I), neither material was absorbed in the intestine and deposited in the liver in appreciable amounts.

When cyanocobalamin and coenzyme were given alone to 3 normals, or with IF preparation to the 3 pernicious anemia patients, both materials were absorbed in the intestine and deposited in the liver (Fig. 1). In all pernicious anemia cases, hepatic uptake of cyanocobalamin, when given with IF, was in

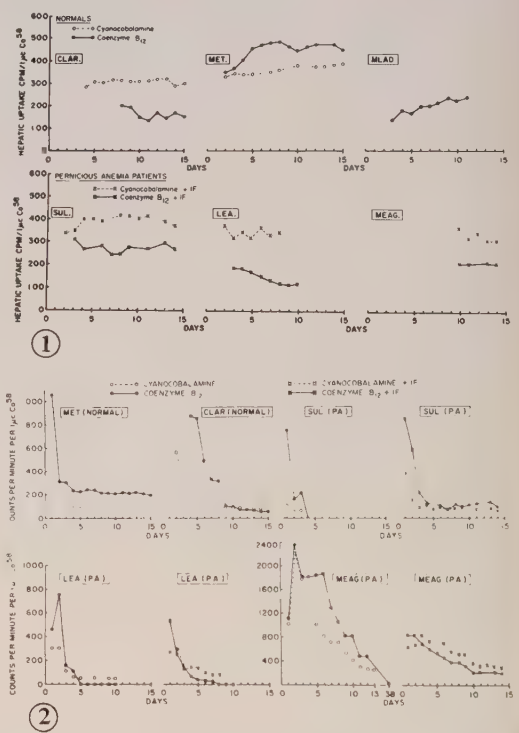


FIG. 1. Hepatic uptake of radioactivity in normals and pernicious anemia patients following oral administration of Co^{58} labelled cyanocobalamine and coenzyme B_{12} alone and together with intrinsic factor (IF).

FIG. 2. Radioactivity counts over intestine in 2 normal and 3 pernicious anemia patients (P.A.) following oral administration of Co^{58} labelled cyanocobalamine and coenzyme B_{12} alone and together with intrinsic factor (IF).

a range similar to that observed in the normal subjects who had received a similar dose of cyanocobalamin without the additional source of IF (Fig. 1). In these pernicious anemia patients, the range of hepatic uptake

was from 300 to 400 cpm per 1 μc of $Co^{58}B_{12}$, which is the normal range of hepatic uptake of $Co^{58}B_{12}$ observed previously (11).

Hepatic uptake of the labelled coenzyme, when given with IF to the same pernicious anemia patients, was only 36-64% of that observed following a similar dose of cyanocobalamin with the same amount of the same IF preparation. Its range was 109-254 cpm per 1 μc Co^{58} as compared to 316-397 cpm per 1 μc Co^{58} hepatic uptake of cyanocobalamin.

As shown by the data listed in Fig. 2, the surface counts over the intestinal projections of normals and patients with pernicious anemia after administration of coenzyme alone were much higher and more prolonged than after administration of corresponding doses of cyanocobalamin. The differences were especially marked during the first few days after administration of the material, but in some instances they extended over the entire 2-week period of observation. After administration of coenzyme together with IF preparation to PA patients, the differences between cyanocobalamin and coenzyme in intestinal counts were less consistent, however, and were seen in only one of the 3 cases tested. In most instances, prolonged intestinal counts masked hepatic uptake for a much longer time than when labelled cyanocobalamin was administered.

Discussion. These results indicate that orally administered coenzyme B_{12}, in the absence of intrinsic factor, is not absorbed in the intestine and therefore cannot be used alone and orally in treatment of pernicious anemia. Moreover, it appears that intestinal absorption of orally administered coenzyme B_{12} in pernicious anemia is less influenced by intrinsic factor preparations derived from animal sources than is that of cyanocobalamin. This occurs in spite of its entering into a bond with these IF preparations(12). Consequently, when these materials are given orally with intrinsic factor preparations from hog stomach to pernicious anemia patients, less coenzyme than cyanocobalamin is deposited in the liver.

Since, in rats, the affinity of coenzyme to the liver is greater than that of cyanocobalamin(4,6), the smaller hepatic deposition of coenzyme in pernicious anemia patients, when administered orally with intrinsic factor preparations, is probably not due to its having less affinity to the liver than cyanocobalamin. There is also no reason to assume that coenzyme exerts a cathartic effect upon the intestine resulting in its more rapid removal from the gut. The smaller hepatic deposition of coenzyme, as compared to cyanocobalamin, is therefore possibly due to its relatively less efficient absorption in the intestine. The same is known to be true for other B_{12} derivatives and analogues as well, such as chloro-, sulpho- or nitrocobalamin (13), or 5,6-dichlorobenzimidazole, 5,6-desdimethylbenzimidazole and 5-hydroxybenzimidazole(14).

In normal subjects, the radioactivity over the intestine was more prolonged after oral administration of labelled coenzyme alone than after cyanocobalamin. The same was also observed in pernicious anemia patients in spite of the fact that no intestinal absorption of this material occurred in absence of IF. This finding may indicate that the attachment of coenzyme to the intestinal mucosa prior to its absorption is easier and stronger than that of cyanocobalamin. This attachment may not depend on the ultimate fate of coenzyme, *i.e.*, its passage through the intestinal mucosa (in normals) or its return to the intestinal lumen (in PA patients).

Summary and conclusions. Measurements of the hepatic uptake of orally administered Co^{58}-labelled 5,6-dibenzimidazolylcobamide coenzyme suggest that it cannot be absorbed in the intestine in the absence of intrinsic factor and, consequently, cannot be used alone in oral treatment of pernicious anemia, Under similar conditions, less coenzyme than cyanocobalamin is deposited in the liver of patients with pernicious anemia when it is orally administered with active IF preparations from animal stomach. This may be due either to the lessened effect of animal IF preparations on intestinal absorption of coenzyme or to the diminished efficiency of in-

testinal absorption of coenzyme as compared with that of cyanocobalamin.

Surface counting of intestinal areas after oral administration of labelled coenzyme alone and labelled cyanocobalamin to normal subjects and patients with pernicious anemia shows the radioactivity of the intestine to be much more prolonged after administration of coenzyme than after cyanocobalamin. This may be interpreted as indicating that, of the 2, coenzyme forms the easier and stronger attachment to the intestinal mucosa prior to its absorption, regardless of whether in the presence of intrinsic factor it ultimately passes through the intestinal mucosa or, in the absence of intrinsic factor, returns to the intestinal lumen.

1. Barker, H. A., Smith, R. D., Weissbach, H., Toohey, J. I., Ladd, J. N., Volcani, B. E., *J. Biol. Chem.,* 1960, v235, 480.

2. Wasserman, L. R., Estren, S., Brody, E., Herbert, V., *Lancet,* 1960, v1, 173.

3. Gräsbeck, R., Bjorksten, F., *ibid.,* 1960, v1, 337.

4. Wong, V., Yeh, S., Chow, B. F., *Fed. Proc.,* 1960, v19, 415.

5. Okuda, K., Chow, B. F., *ibid.,* 1960, v19, 417.

6. Uchino, H., Ukyo, S., Wakisaka, G., Proc. 8th Internat. Congr. Hematol., Tokyo, Sept. 1960, in press.

7. Barbee, K. W., Johnson, B. C., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 720.

8. Jasinski, B., Stiefel, G. E., Frei, H., Wuhrmann, F., *Clin. Chim. Acta,* 1956, v1, 189.

9. Glass, G. B. J., Boyd, L. J., Gellin, G. A., Stephanson, L., *Arch. Biochem.,* 1954, v51, 251.

10. Glass, G. B. J., Boyd, L. J., Gellin, G. A., *Blood,* 1955, v10, 95.

11. Gran, V., Glass, G. B. J., *J. Lab. and Clin. Med.,* 1961, v57, 562.

12. Ellenbogen, L., Highley, D. R., Barker, H. A., Smyth, R. D., *Biochem. and Biophys. Res. Communic.,* 1960, v3, 178.

13. Rosenblum, C., Yamamato, R. S., Wood, R., Woodbury, D. T., Okuda, K., Chow, B. F., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 364.

14. Rosenblum, C., Davis, R. L., Chow, B. F., *ibid.,* 1957, v95, 30.

Received March 10, 1961. P.S.E.B.M., 1961, v107.

Lipoprotein Pre-Staining and Ultracentrifugal Analysis in a Density Gradient.* (26607)

DAVID G. CORNWELL AND FRED A. KRUGER (Introduced by Chauncey D. Leake)
Dept. of Physiological Chemistry and Division of Endocrinology and Metabolism, Dept. of Medicine, Ohio State University, Columbus

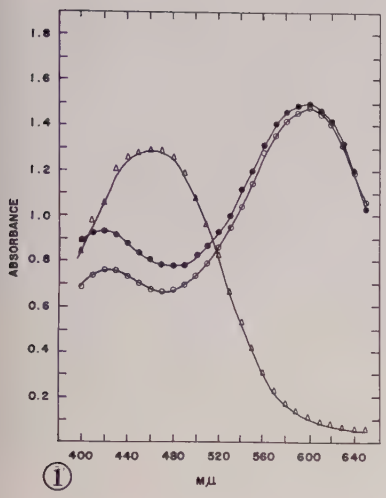
Pre-staining methods, used for analysis of serum lipoproteins by paper electrophoresis (1-4) and ultracentrifugal flotation(5,6), are not quantitative. Lipids dispersed on celite have been solubilized by agitation with serum lipoproteins(7). A modified procedure, pre-staining with a sudan black B (SBB) - celite slurry, is used here for the ultracentrifugal analysis of serum lipoproteins in a density gradient(8).

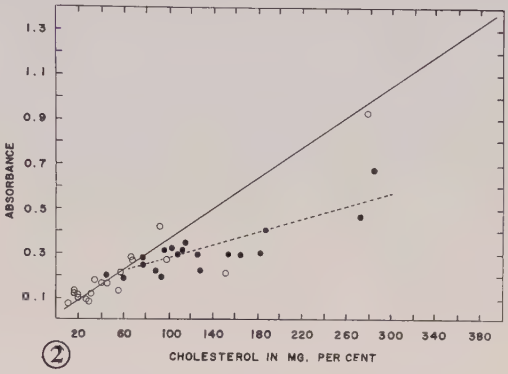
Methods. SBB - celite slurry. Two g SBB† was dissolved in 200 ml methyl cellosolve and added to 10 g celite 535. Distilled water, 500 ml, followed by 100 ml 2 *M* NaCl were then added with constant stirring. The suspension was filtered on a sintered glass filter, the cake washed with 300 ml 0.15 *M* NaCl, transferred to a glass stoppered flask, and suspended in 100 ml 0.15 *M* NaCl. *Staining.* Three ml of thoroughly mixed slurry was added to 1 ml of serum in a test-tube and agitated gently on an automatic shaker for 15 hr at room temperature. The tube was then centrifuged for 30 min at 1000 $\times$ *g* and stained diluted serum withdrawn.

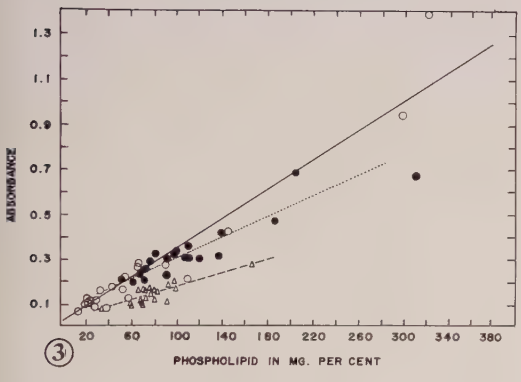
Ultracentrifugation. Three ml of the stained serum was placed in a 13.5 ml centrifuge tube containing 1 ml 2 *M* NaCl and mixed. Five ml 0.15 *M* NaCl was layered over this solution and the tube centrifuged

* Supported in part by Research Grants from Nat. Heart Inst., P.H.S.

† Purchased from National Aniline Div., Allied Chem. and Dye Corp., New York.







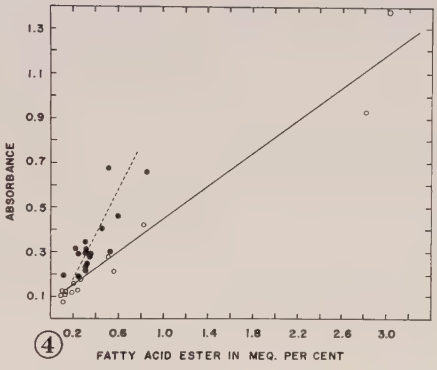


FIG. 1. Absorbance of dye solutions in ethanol: SBB, ○; SBB from slurry, ●; polar dye from filtrate, △.

FIG. 2. SBB absorbance of lipoprotein fractions as a function of cholesterol content: S_f 10-400, ○; S_f 0-10, ●.

FIG. 3. SBB absorbance of lipoprotein fractions as a function of phospholipid content: S_f 10-400, ○; S_f 0-10, ●; high density, △.

FIG. 4. SBB absorbance of lipoprotein fractions as a function of FAE content: S_f 10-400, ○; S_f 0-10, ●.

at 9,300 × *g* for 30 min in a Spinco Model L ultracentrifuge. The top chylomicron layer was removed with a tube cutter and infranatant serum layered over 5.5 ml 2.5 *M* NaCl in a centrifuge tube. Traces of SBB-celite adhering to the tube were not transferred. The gradient tube was filled with 0.15 *M* NaCl and centrifuged at 100,000 × *g* for 20 hr. Three stained lipoprotein fractions—the top S_f 10-400, middle S_f 0-10, and bottom high density lipoproteins—were transferred to 25 ml volumetric flasks. Ethanol was added, the flasks heated to boiling, cooled and diluted to volume with ethanol. The contents were filtered through Whatman No. 42 paper and SBB absorbance read at 600 mμ in a Beckman Model B spectrophotometer. In control experiments, lipoproteins were isolated by density gradient ultracentrifugation and analyzed for cholesterol, phospholipid, and fatty acid ester (FAE) content(8).

Results. Absorbancies in ethanol of the original SBB, SBB precipitated on celite, and dye from a celite cake filtrate are recorded in Fig. 1. A water soluble dye with an absorbance maximum at 460 mμ is found in the filtrate. The absorbance maximum for SBB is at 600 mμ. Reproducible stain uptake was obtained with typical replicate

TABLE I. Cholesterol and Sudan Black B Distribution in Serum and Serum Lipoproteins of a Hyperlipemic Diabetic Subject on Insulin and Unsaturated Fatty Acid Therapy.

Fraction and analysis	Date 1/11/60	1/15/60	2/1/60
Serum			
Cholesterol, mg %	508	465	268
SBB absorbance*	1.810	1.485	.779
Chylomicron			
Cholesterol, mg %	19.1	54.4	10.9
SBB absorbance	.097	.211	.054
S_f 10-400			
Cholesterol, mg %	371	279	92.5
SBB absorbance	1.380	.928	.420
S_f 0-10			
Cholesterol, mg %	45.0	77.5	90.0
SBB absorbance	.200	.249	.222
High density			
Cholesterol, mg %	28.0	27.0	30.4
SBB absorbance	.132	.099	.084

* Sum of SBB absorbancies in different fractions.

analyses of 0.574 ± 0.033, 0.441 ± 0.019, and 0.481 ± 0.014 for different serum pools. Stain intensity varied with the slurry preparation. One ml aliquots from a serum pool were stored at –20°C for several months with no change in SBB uptake from a given slurry and were used to standardize different slurry preparations. Stain intensity increased with agitation time. Reproducibility and proportional stain uptake improved when agitation was continued for 15 hr.

In Fig. 2-4, SBB uptake for lipoproteins isolated from subjects with widely varying lipoprotein concentrations is compared with their cholesterol, phospholipid, and FAE content. Table I illustrates the usefulness as well as the shortcomings of the SBB staining technic in following total serum lipids and individual lipoprotein concentrations in hyperlipemic subjects during therapy. Total cholesterol, as well as chylomicron and S_f 10-400 lipoprotein cholesterol, correlate well with staining intensity whereas S_f 0-10 and high density lipoprotein cholesterol do not.

Discussion. Stain intensity and reproducibility are difficult to control when SBB dissolved in organic solvents is added to serum (5,6). Total dye uptake is often similar for sera of widely varying lipid content. Staining with SBB-slurry more nearly reflects total lipid concentration. However, SBB uptake varies with different lipoproteins and is not a simple measure of total lipid. Individual lipid components differ considerably in their affinity for stain. Berg *et al.*(9) have shown that although staining of any given lipid by SBB on paper is proportional to the amount of lipid, individual lipids differ widely in their capacity to retain dye. Triglycerides stain most intensely, while equal weights of cholesterol, phospholipid, and cholesterol ester take up considerably less SBB. Since lipoproteins differ markedly in lipid content, those containing relatively large amounts of triglyceride (elevated FAE), the chylomicrons and S_f 10-400 lipoproteins, stain proportionately more for a given amount of lipid and correlate with lipoprotein concentration (Table I). Since S_f 0-10 lipoproteins contain relatively less triglyceride, and since the relative FAE content of this fraction is enhanced in hyperlipemia when the absolute concentration of the fraction is suppressed(8), staining correlates poorly with concentration of S_f 0-10 lipoproteins in hyperlipemia (Table I). Elevations in S_f 0-10 lipoproteins with a normal lipid composition, as found in myxedema or idiopathic hypercholesterolemia, may be satisfactorily estimated by this procedure. Prestaining is a useful adjunct in flotation studies with other animal sera as well as lipoproteins from other sources such as egg yolk since the lipid containing fractions are readily visualized in appropriate density gradients.

Celite slurry increases surface area between non-polar dye and lipoproteins in solution. Precipitation on celite by increasing solvent ionic strength results in highly dispersed SBB-celite, enhancing staining more than celite dispersions prepared by solvent evaporation(7). Serum aliquots may be stored at –20°C without major changes in lipoprotein properties(10) and used as standards for different slurry preparations. The polar dye found in filtrates during slurry preparations is probably the SBB decomposition product(11) capable of staining

more polar lipids(12) and proteins(13). SBB may be purified by repeated solvent precipitation from a methyl cellosolve solution with saline.

Summary. The preparation and standardization of a SBB - celite slurry for lipoprotein pre-staining is described. Dye uptake is proportional to lipid concentration and may be used to estimate chylomicrons, S_f 10-400 and S_f 0-10 lipoproteins isolated by density gradient ultracentrifugation. Precipitation on celite by altering solvent polarity is an effective means for preparing highly dispersed SBB and other lipid soluble substances. A polar dye contaminant is removed from SBB by solvent precipitation.

1. McDonald, H. J., Bermes, E. W., *Biochim. Biophys. Acta,* 1955, v17, 290.

2. Solinas, P., Betti, R., Di Leo, E. F., *Clin. Chim. Acta,* 1957, v2, 586.

3. McDonald, H. J., Ribeiro, L. P., *ibid.,* 1959, v4, 458.

4. McDonald, H. J., Kissane, J. Q., *Anal. Biochem.,* 1960, v1, 178.

5. Cornwell, D. G., Kruger, F. A., *Abst. 135th Meet. Am. Chem. Soc.,* 1959, 35c.

6. Light, S., Gurd, F. R. N., *Vox Sanguinis,* 1959, v5, 92.

7. Avigan, J., *J. Biol. Chem.,* 1959, v234, 787.

8. Cornwell, D. G., Kruger, F. A., Hamwi, G. J., Brown, J. B., *Am. J. Clin. Nutrition,* 1961, v9, 24, 41.

9. Berg, G., Scheiffarth, F., Estler, C. J., Schön, H., *Klin. Wochen.,* 1958, v36, 766.

10. Briner, W. W., Riddle, J. W., Cornwell, D. G., PROC. SOC. EXP. BIOL. AND MED., 1959, v101, 784.

11. Fredricsson, B., Laurent, T. C., Luning, B., *Stain Techn.,* 1958, v33, 155.

12. Diezel, P. B., Niemanis, G., *Virchow's Archiv.,* 1957, v330, 619.

13. Kutt, H., Lockwood, D., McDowell, F., *Stain Techn.,* 1959, v34, 203.

Received March 14, 1961. P.S.E.B.M., 1961, v107

Effect of Reserpine on Lipoprotein Lipase Activity of Rat Heart. (26608)

ABRAHAM DURY

General Medical Research, Veterans Administration Hospital and Department of Anatomy, School of Medicine, University of Pitssburgh, Pittsburgh, Pa.

The clearing factor lipase in post-heparin plasma and in extracts of adipose and heart tissue was shown to be similar and named lipoprotein lipase(1-3). It generally is believed(4,5) that lipoprotein lipase plays a role in transfer of fat from the circulating blood into tissues and mobilization of fat from adipose tissue. Although heparin-activated release of lipoprotein lipase from peripheral tissues is well documented(5-8), the physiologic control of activation and release of lipoprotein lipase is relatively unknown. Studies by Hollenberg(9,10) and Cherkes and Gordon(11) have indicated that activity of lipoprotein lipase in heart and adipose tissue is related to nutritional state of the rat. However, fasting induced opposite effects on the activity of lipoprotein lipase in heart and fat tissue suggesting the involvement of other factors. Another mode of control, possibly more direct, of the activity of lipoprotein lipase is by hormonal or neurohumoral mechanisms. Several types of hormones markedly influence the level of plasma free fatty acids (FFA) and effect release of FFA from adipose tissue(4,12). In this connection, Wadstrom(13) and White and Engel(14) have shown that hydrolysis of triglycerides in adipose tissue was stimulated by epinephrine and norepinephrine *in vivo* and *in vitro*.

The work reported here was done on rat myocardial tissue because it normally contains a relative abundance of norepinephrine and epinephrine(15) which are rapidly depleted following injection of reserpine(16). Also, the activity of lipoprotein lipase in rat heart has been shown responsive to a physiological stimulus(10). This paper reports the effect, in rats, of reserpine pretreatment on the amount of lipoprotein lipase and its release by heparin from heart tissue *in vitro*.

Materials and methods. Female Wistar rats weighing about 200 g were injected intraperitoneally with Reserpine (Serpasil, Ciba), 0.2 mg/100 g, either once—Group A, or once daily for 4 days—Group B. Controls were similarly treated with 0.5 ml of isotonic saline. Food was removed at time of the last injection, and 20 hours later rats were sacrificed under ether anesthesia. The heart was excised, cut open with scissors, quickly rinsed in ice-cold Ringer's solution, and sliced with a razor blade. Tissue, weighing 150-170 mg, from each heart was placed into each of a pair of 25 ml Erlenmeyer flasks containing 3.0 ml of Krebs-Ringer phosphate buffer, pH 7.4. To one flask of a pair was added 0.2 mg (0.2 ml) of heparin (Heparin, Upjohn, diluted with water) while an equal amount of distilled water was added to the other flask. Heart slices were agitated in a Dubnoff metabolic incubator at 37°C, under air, for 45 minutes. Heart slices from control and reserpine pretreated rats were incubated at the same time. The activity of the lipase released into the incubation medium was measured by its ability to produce FFA from an activated coconut oil substrate. The substrate mixture was made up as follows: 1 part of fresh human serum; 1 part of a 5% coconut oil emulsion (Ediol, Schenlabs) prepared by dilution with water; and 8 parts of freshly prepared 10% solution of bovine serum albumin adjusted to pH 7.4. The albumin was a dialyzed and lyophilized stock prepared from BSA powder, Fraction V purchased from Nutritional Biochemicals, Inc. Portions (0.5 ml) of the substrate mixture were pipetted into 15 ml glass-stoppered centrifuge tubes and incubated at 37°C for 30 minutes prior to mixing with 0.5 ml aliquots, taken in duplicate, of the incubation medium from each flask. After incubating the entire reaction mixture for 1 hour at 37°C, the FFA produced was determined by the method of Dole(17) using Nile Blue indicator solution. Substrate blanks were run with each series of incubated heart slices. The activity of the lipase in the medium is expressed as micro-equivalents of FFA produced/g heart/hour.

To demonstrate that the lipolytic enzyme released from heart tissue was similar to lipoprotein lipase, the effects of protamine sulfate and 1M sodium chloride in the incubating medium were tested. Heart slices from 16 normal, 20-hour fasted rats were processed as described above, and to each incubation flask was added 0.2 mg of heparin. For inhibition by protamine, 50 mg of protamine sulfate was added to one flask of a pair at exactly 40 minutes of elapsed incubation. For inhibition by 1M sodium chloride, the medium of one flask of a pair contained 58.5 mg/ml of the salt. The contents of all flasks were incubated for a total of 45 minutes. Aliquots, in duplicate, of each incubation medium were mixed with activated coconut oil substrate, incubated for another hour, and lipase activity determined as described above.

Results. Lipolytic activity from normal heart tissue. Release of lipolytic activity was the same with control heart tissue from normal *ad lib.* fed and 20-hour fasted rats. The effect of heparin was to increase similarly the lipolytic activity of heart slices from both types of rats (Fig. 1).

The data presented in Table I demonstrate inhibition of lipolytic activity in the incubating medium when protamine sulfate and 1M sodium chloride, known inhibitors of lipoprotein lipase, were added to the heart slices. Inhibition of enzyme activity by 1M sodium chloride was almost complete while protamine caused a slightly lesser degree of inhibition. These data are taken to indicate that the lipase released into the medium from heart tissue was identical with lipoprotein lipase.

Lipolytic activity from heart slices of reserpine-treated rats. The amounts of lipase released from control heart tissue of Groups

TABLE I. Inhibition of Lipase Liberated by Heart Tissue of Normal, Fasted Rats.

Inhibitor	Inhibition of lipolysis (%)
Protamine sulfate	76.9 (54.9- 87.5)*
1M NaCl	93.6 (87.9-100.0)*

* Mean and range, 8 tissues.
See *Methods* for details.

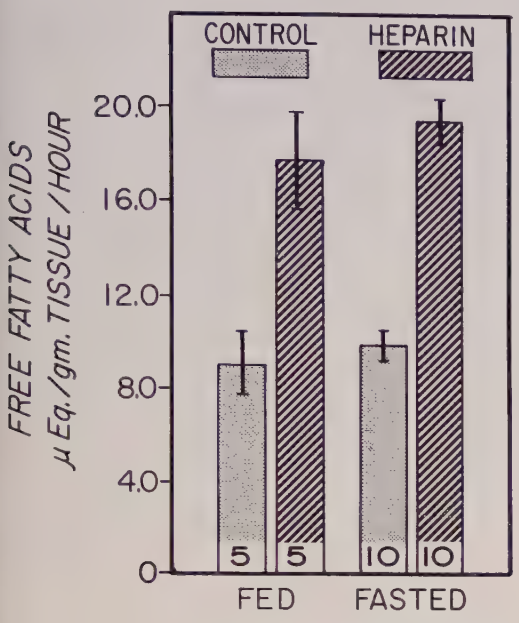


FIG. 1. Release of lipoprotein lipase from incubated heart tissue of normal fed and 20-hour fasted rats. Number at bottom of each column indicates No. of animals in group. Vertical bars equals S.E.

A and B were 63% and 43%, respectively, of the activity released by myocardial slices from normal fasted rats (Table II). The effect of adding heparin to the incubating medium also is shown in Table II. The activity determined in the medium with normal rat heart was 100% greater than without added heparin. In the reserpine pretreated groups, the effect of heparin was an 80% increase in lipolytic activity when heart slices were used from Group A; in experiments with heart tissue from Group B, heparin did not significantly change lipolytic activity of the medium. Furthermore, with heart slices of both reserpine pretreated groups, the absolute amount of activity released by heparin was considerably below that found with normal rat heart. These results suggest that the marked decrease in release of lipolytic activity from heart slices in reserpine-treated rats was due to a primary influence of reserpine on the amount of lipoprotein lipase in myocardial tissue.

TABLE II. Effect of *In Vivo* Administered Reserpine on Lipoprotein Lipase Release by Heart Tissue from Fasted Rats.

Heparin added to medium	Lipase activity released† Controls	Reserpine inj.
None	9.79 ± .62	(A) 6.20 ± .51* (B) 4.28 ± .53*
.2 mg	19.45 ± .82	(A) 11.24 ±1.95* (B) 6.40 ± .79*
	Heparin-induced release of lipase, value of P =	
	.001	(A) .035 (B) NS

Reserpine, I.P., 0.1 mg/100 g of rat. (A) = one injection; (B) = one injection a day for 4 days. All animals fasted 20 hr before sacrificed.

* Difference between control and reserpine tissues occurring by chance alone, P = <0.01.

† Lipolytic activity expressed as μEq. of FFA/g tissue/hr. Controls: 10 tissues; Reserpine: (A) 6 tissues, (B) 4 tissues.

Discussion. The results obtained with normal *ad lib.* fed and 20-hour fasted rat heart slices indicated that such difference in the nutritional state of the donor rat was not important in determining the amount of lipoprotein lipase released without and with heparin during incubation of heart tissue. Hollenberg(10) and Cherkes and Gordon (11) also had observed that a short fasting period did not alter the heparin effect on lipolytic activity of heart slices. On the other hand, with a longer period of starvation (3-4 days) heparin profoundly increased the activity of the enzyme released from heart slices(10). The reason for this result is not clear.

The evidence presented here indicates that heart lipoprotein lipase activity is reduced following one or more injections of reserpine into rats. Moreover, heparin failed to elicit a significant increase of the activity when heart tissue was used from rats pretreated with reserpine for 4 days, suggesting a primary influence of reserpine on amount of lipoprotein lipase in myocardial tissue.

The mode of action of reserpine is not indicated in this study. However, one possibility is that the reduced lipoprotein lipase activity in myocardial tissue is related to reserpine-induced depletion of stores of heart catecholamines. Paasonen and Krayer (16) have shown that the effect of reserpine on heart tissue catecholamines was remarkably similar to the disappearance of neuro-

hormones from the myocardium due to degeneration of adrenergic nerves. A working hypothesis is that the activity of lipoprotein lipase is stimulated by free (or "released") norepinephrine and/or epinephrine in heart tissue. In this connection, it was found (studies in progress) that pretreatment of rats with monoamine oxidase inhibitors (Marplan and Iproniazid) increased lipoprotein lipase activity released from heart tissue and the effect of heparin on enzyme activity was increased 2-fold.

Summary. The lipoprotein lipase activity released from heart slices of normal *ad lib.* fed and 20-hour fasted rats was the same. Heparin elevated equally the activity of tissues from both types of rats. Pretreatment with reserpine reduced the amount of activity of lipoprotein lipase released by incubated heart slices. The effect of heparin was negligible when heart tissues were used from rats treated with reserpine once daily for 4 days. The possibility of a relationship between reserpine-induced decrease in activity of lipoprotein lipase and depletion of catecholamines from heart tissue was discussed.

1. Korn, E. D., *J. Biol. Chem.,* 1955, v215, 1.
2. ———, *ibid.,* 15.
3. Korn, E. D., Quigley, T. W., Jr., *Biochem et Biophys Acta,* 1955, v18, 143.
4. Frederickson, D. S., Gordon, R. S., Jr., *Physiol. Revs.,* 1958, v38, 585.
5. Robinson, D. S., *Am. J. Clin. Nutrition,* 1960, v8, 7.
6. Shore, B., Nichols, A. V., Freeman, N. K., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 216.
7. Jeffries, G. H., *Quart. J. Exp. Physiol.,* 1954, v39, 261.
8. Robinson, D. S., Harris, P. M., *ibid.,* 1959, v44, 80.
9. Hollenberg, C. H., *Am. J. Physiol.,* 1959, v197, 667.
10. ———, *J. Clin. Invest.,* 1960, v39, 1282.
11. Cherkes, A., Gordon, R. S., Jr., *J. Lipid Res.,* 1959, v1, 97.
12. Engel, F. L., White, J. E., *Am. J. Clin. Nutrition,* 1960, v8, 691.
13. Wadstrom, L. B., *Nature,* 1957, v179, 259.
14. White, J. E., Engel, F. L., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 375.
15. Von Euler, U. S., *Acta Physiol. Scand.,* 1946, v11, 168.
16. Paasonen, M. K., Krayer, O., *J. Pharm. & Exp. Therap.,* 1958, v123, 153.
17. Dole, V. P., *J. Clin. Invest.,* 1956, v35, 150.

Received March 15, 1961. P.S.E.B.M., 1961, v107.

Nucleic Acid Synthesis by Leukocytes in Presence of Anti-Leukocyte Factors.* (26609)

ANNA MARIA WILLIAMS AND ROBERT F. SCHILLING
Department of Medicine, University of Wisconsin Medical School, Madison

A variety of evidence(1) indicates reactions between antinuclear substances in sera from patients with systemic lupus erythematosus and cell nuclei, nucleoprotein, and DNA.† There appear to be at least 2 factors: one which reacts with cell nuclei and is responsible for the "L.E. phenomenon," and another, present only in certain sera, which reacts with purified DNA from different sources and species. Both factors migrate with the gamma globulins on zone electrophoresis, and it has been hypothesized that the described interactions are antigen-antibody reactions.

Calabresi, Edwards, and Schilling(2), using a fluorescent antibody technic, have detected antinuclear globulins in the sera of all patients with SLE or Felty's syndrome of rheumatoid arthritis which they studied. They also obtained evidence, by plasma transfusions, that a leukopenic factor was present in the plasma from 2 patients with

* This research was supported by a grant from U.S.P.H.S.

† The following abbreviations are used: DNA, deoxyribonucleic acid; SLE, systemic lupus erythematosus; RNA, ribonucleic acid; CGL, chronic granulocytic leukemia.

Felty's syndrome. In conjunction with their work, we have tested some SLE and Felty sera for possible effects, as compared to normal sera, on DNA, RNA, and protein synthesis by human leukocytes. To evaluate the results, we also compared normal rabbit serum and rabbit serum containing agglutinins to human leukocytes for effects on synthesis by human leukocytes.

Materials and methods. Sera: To minimize any effects of different salt concentrations, sera to be used in incubations with leukocytes were dialyzed for two 2-hour periods against 100× volume portions of the glucose-salts incubation mixture.

Incubations with human leukocytes. Leukocytes (number indicated in tables) obtained from patients with chronic granulocytic leukemia were incubated for 1 hour under 95% air + 5% CO_2 in Robinson's salts mixture(3) supplemented with 100 mg% glucose and 209 mg% sodium bicarbonate. Experiments with 25% serum were carried out in 60-ml Warburg vessels containing 1.0 μM radioactive precursor in 12.0 ml medium or in 9.0 ml medium + 3.0 ml experimental serum. Experiments with 100% serum were carried out in 25-ml Warburg vessels containing 0.5 μM radioactive precursor in 3.0 ml medium or serum. Methodology for preparation and incubation of leukocyte suspensions and separation of nucleic acid fractions has been described(4). Differences in the present procedure are as follows: Instead of perchloric acid addition immediately after incubation, contents of the chilled flasks were centrifuged in the cold and the sera were poured off. In measuring incorporations of glycine-2-C^{14} into cell protein, the pellets were washed once with a 10× volume of cold nonradioactive medium and recentrifuged before addition of perchloric acid. Specific activity of all radioactive compounds was 2×10^6 cpm/μM.

Preparation of rabbit antileukocyte serum. Three rabbits were immunized for another study by injection of human leukocytes prepared from (1) chronic myelogenous leukemic, (2) chronic lymphocytic leukemic, and (3) normal blood. Each rabbit was given an intravenous injection of 1.5×10^6 leukocytes and a subcutaneous injection of 3.5×10^7 leukocytes one month later. Ten ml immune serum from each rabbit were harvested 2 months after initial injection and pooled for the present study. After inactivation at 56°C for 30 minutes, the serum was incubated with an equal volume of washed human red cells in a 37°C water bath with gentle agitation for 2 hours, and then at 4°C for 8 hours, after which serum and red cells were separated by centrifugation. Normal rabbit serum was treated by the same procedure. The sera were then dialyzed against the glucose-salts incubation mixture as described above.

Results and discussion. The effects of 25% normal serum are shown in Table I, Exp. 1 and 2. Compared to the control without serum, incorporation of adenine-8-C^{14} into RNA adenine was higher, into RNA guanine lower or unchanged, into DNA adenine lower, and incorporation of thymidine into DNA was lower. Serum from a patient with chronic granulocytic leukemia, one with SLE, and one of 2 patients with rheumatoid arthritis showing Felty's syndrome had the same effects as normal sera. Another serum from a patient with Felty's syndrome gave an inhibition instead of a stimulation of adenine incorporation into RNA adenine (but no marked effect as compared to the control), and a greater inhibition of thymidine incorporation into DNA than did the normal serum tested in the same experiment (although not greater than the inhibition by the normal serum of Exp. 3). This sample was also run undialyzed as a check on possible loss of activity upon dialysis, but values did not differ greatly from those with the dialyzed serum.

In Exp. 3, Table I, a smaller number of cells was suspended in 3.0 ml undiluted serum, thus increasing the ratio of inhibitory or stimulatory factors to cells. Serum (or glucose-salts control) was preincubated with cells for 30 minutes at 3°C before addition of radioactive precursor. As with 25% serum, incorporation into RNA was higher and into DNA lower than the control, and results

TABLE I. Effect of Human Sera on Incorporation by Human Leukocytes from CGL.

	Serum	Specific activity with serum * / Specific activity without serum: Adenine-8-C^{14} RNA adenine	Adenine-8-C^{14} DNA adenine	Adenine-8-C^{14} RNA guanine	Thymidine-H^3 DNA
Exp. 1: 25% serum in 12.0 ml incubation medium; 5×10^8 cells C.L.	F.S. Normal	1.31	.87	.44	—
	A.W. "	—	—	—	.73
	D.C. CGL	1.16	.71	1.00	—
	R.S. Felty	.82	.91	.46	.37
	" " undialyzed	.86	.98	.57	.48
Exp. 2: 25% serum in 12.0 ml incubation medium; 5×10^8 cells A.M.	F.S. Normal	1.17	.64	.76	—
	S.C. "	1.32	.51	.99	.72
	I.B. Felty†	1.13	.48	1.01	.77
	A.T. SLE	—	—	—	.88
Exp. 3: 100% serum in 3.0 ml incubation medium; 2×10^8 cells S.C.; cells & serum pre-incubated 30 min. at 3°C before addition of radioactive precursor	R.W. Normal	1.30	.52	—	.39
	I.B. Felty†	1.35	.37	—	.50

* Specific activity values were averages of duplicate flasks which varied by less than 10% from the mean.

† Also positive LE preparation.

with a SLE serum were similar to those with normal serum.

The experiments shown in Table I tested a few sera with each of 3 suspensions of leukocytes from 3 different patients. To determine the variation in effect of a larger number of sera on one leukocyte population, the experiment shown in Table II was performed.

TABLE II. Effect of Human Sera on Incorporation of Adenine-8-C^{14} by Human Leukocytes from CGL.

Serum	Sp. act. with serum * / Sp. act. without serum	
Incubation 1†		
J.S. Normal	1.10	1.04
J.V. "	.98	1.03
P.P. SLE (4.1)‡	1.03	1.18
R.S. Felty (1.7)	1.04	1.09
Incubation 2§		
L.S. Normal	1.13	1.27
M.E. "	.96	1.30
E.B. Felty (1.6)	1.10	1.17
D.W. Felty (1.6)	1.13	1.31

* Specific activity values were avg of duplicate flasks which varied by less than 10% from the mean.

† Flasks without serum: RNA adenine = 26,430 ± 210 cpm/μM; DNA adenine = 480 ± 22 cpm/μM.

‡ Wbc ($\times 10^3$/mm^3) when serum was withdrawn.

§ RNA adenine = 28,740 ± 1360; DNA adenine = 563 ± 6.

Sera from 4 normal subjects, 3 leukopenic patients with Felty's syndrome, and 1 patient with SLE were tested with leukocytes from G.Z. under the conditions described for Exp. 3, Table I. Blood was withdrawn from G.Z. twice on the same day for the 2 incubations, each of which included 2 normal and 2 pathological sera. As in the previous studies, pathological sera had no marked effects over those from normal sera. In this experiment, incorporation into both RNA and DNA adenine with serum was either somewhat higher than the control or was not significantly different.

Frenster *et al.*(5) have reported a decrease in rate of incorporation of formate and glycine into the protein plus nucleic acid fraction of human leukemic leukocytes when normal or pathological human serum was added to the incubation mixture. In 3 experiments of the present study, there was an inhibition or no marked change of incorporation at 60 minutes into RNA guanine and into DNA, but a stimulation of that into RNA adenine. In one experiment, incorporation into both RNA and DNA adenine either was not significantly affected or was stimulated by serum. Thus, results with both normal and pathological sera were variable. It was

TABLE III. Effect of Rabbit Serum Containing Agglutinins to Human Leukocytes on Incorporation by Human Leukocytes from CGL.

Cells B. G. 3.0 ml medium	Glycine-2-C^{14}, 2×10^7 cells, cpm/mg protein	Adenine-8-C^{14}, 4×10^8 cells cpm/μM RNA adenine	cpm/μM DNA adenine	Thymidine-H^3, 10^8 cells, cpm/mg DNA
Glucose-salts	650 ± 42*	19,260 ± 650	459 ± 18	980 ± 60
Normal serum 1:2	562 ± 24	18,260 ± 860	511 ± 15	876 ± 60
Immune " "	546 ± 36	20,960 ± 470	514 ± 14	1164 ± 10
Normal serum 1:50	958 ± 62	18,230 ± 750	706 ± 59	988 ± 96
Immune " "	1150 ± 32	16,760 ± 250	438 ± 8	1052 ± 40

* Variation from mean of duplicate flasks.

somewhat surprising to us that the pathological sera tested did not exert additional effects on *in vitro* incorporation into leukocytes. Plasmas from R.S. and I.B. caused definite, transient depressions of the leukocyte count when transfused into other human recipients (2). Antinuclear globulins were detected, by means of the fluorescent antibody test described in (2), in the sera of all patients with SLE or Felty's syndrome used in the incorporation studies. These antinuclear globulins combined with nuclei of both granulocytes and lymphocytes. Sera were withdrawn for incorporation tests while the patients were in an active state of their disease.

To determine what effect on incorporation sera known to contain antibodies would have, serum was harvested from rabbits immunized against human leukocytes. Normal rabbit serum prepared as described under *Methods* caused no macroscopic or microscopic agglutination when the relative proportions of white cells and serum used in incubations were mixed together. Immune rabbit serum, in dilutions up to 1:10, caused macroscopic agglutination within 15 minutes or less, at 3°C or room temperature. In the incubation experiments, cells and serum (or glucose-salts mixture) were preincubated 30 minutes at 3°C before addition of radioactive precursor. The sera were used at 1:2 and 1:50 dilutions; at the latter dilution, agglutination did not occur with the immune serum. When the vessels containing immune serum in the 1:2 dilution were removed from the shaker, the cells were observed to be agglutinated.

However, results for normal and immune sera differed markedly only for incorporation of adenine-8-C^{14} into DNA with the serum diluted 1:50, where normal serum was stimulatory and immune serum caused no significant change (Table III). At this dilution, there was no significant difference between normal and immune sera for incorporation of thymidine-H^3 into DNA, and at the 1:2 dilution, incorporation of the latter precursor was lower than the control in presence of normal serum and higher in presence of immune serum. There was thus no consistent difference in effect of immune serum compared to normal serum.

Holman *et al.*(1) have reported that the antinuclear factors present in SLE sera did not interfere with growth of normal or tumor cells in tissue culture. From these and other data they concluded that the factors could not gain access to the nucleus of a viable cell. This possible nonpenetration to the nucleus may at least partly explain the failure in the present study to demonstrate an effect of anti-leukocyte factors on incorporation *in vitro*. In addition, since rabbit sera with antibody concentrations which caused macroscopic agglutination did not cause marked differences in incorporation, it perhaps should not be too surprising that no pronounced effects were obtained with the human pathological sera, in which postulated antibodies would be present in lower concentrations.

Summary. Normal and pathological human sera were tested for effects on incorporation of nucleic acid precursors into RNA and DNA during 60-minute incubations. Sera from systemic lupus erythematosus or Felty's syndrome of rheumatoid arthritis did not differ markedly from normal sera. With serum (compared to glucose-salts control),

incorporation of adenine-8-C^{14} into RNA adenine was higher or unchanged, into RNA guanine lower or unchanged, and into DNA adenine variable; incorporation of thymidine-H^3 into DNA was lower. Normal rabbit serum and immune rabbit serum containing agglutinins to human leukocytes were tested for effects on incorporation of precursors into nucleic acids and protein. Immune serum at a concentration causing macroscopic agglutination of the cells gave results similar to those with normal (non-agglutinating) serum.

The authors express their appreciation to Elaine Joranger for technical help, and to Dr. Marjorie Hickman for rabbit serum containing agglutinins for human leukocytes.

1. Holman, H. R., Deicher, H. R. G., Kunkel, H. G., *Bull. N. Y. Acad. Med.,* 1959, v35, 409.
2. Calabresi, P., Edwards, E. A., Schilling, R. F., *J. Clin. Invest.,* 1959, v38, 2091.
3. Robinson, J. R., *Biochem. J.,* 1949, v45, 68.
4. Williams, A. M., Schilling, R. F., *J. Lab. Clin. Med.,* In Press, 1961.
5. Frenster, J. H., Best, W. R., Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 887.

Received March 17, 1961. P.S.E.B.M., 1961, v107.

Studies on Bile Acids in Rat Systemic Blood. Bile Acids and Steroids 107.* (26610)

SCOTT M. GRUNDY† AND JAN SJOVALL (Introduced by A. C. Griffin)
Department of Chemistry, Karolinska Insitutet, Stockholm

For several decades many attempts have been made to estimate blood bile acid levels (Sobotka(1)). The results of these studies have varied greatly and even during the past 10 years values ranging from 0 to 40 mg per 100 ml plasma have been reported(2). Most values have been obtained using direct colorimetric methods of doubtful specificity. During the last few years chromatographic methods have been applied prior to spectrophotometric or fluorometric measurements(3,4). With these methods no bile acids could be detected in normal human serums. Although bile acids have never been isolated from blood, Carey(5) has observed spots corresponding to bile acids on paper chromatograms of purified human serum extracts. He also found through spectrophotometric methods trihydroxy- and dihydroxycholanic acid levels of 0.14 and 0.08 mg/100 ml respectively(6).

Byers and Friedman have reported the cholate level in rat serum to be 2.1 mg/100 ml(7). Since bile acid metabolism has been studied extensively in the rat we have further investigated the bile acids in the systemic blood of this animal. This work is a continuation of a previous study of the portal bile acids(8).

Experimental. Cholic acid was labelled by exposure to one curie of tritium gas(9) for 14 days in the apparatus described by Bergström and Lindstedt(10). To remove labile tritium the cholic acid was repeatedly evaporated with ethanol-water, then heated for 16 hours at 120°C in 2 N NaOH. After ether extraction of the acidified solution the acid was chromatographed twice with phase system C described below. The cholic acid peak was diluted with twice the weight of inactive cholic acid and recrystallized twice giving a specific activity of about 6.9×10^8 cpm per mg when counted in an infinitely thin layer in a methane gas-flow counter (Frieseke Hoepfner FH 51).

Sprague-Dawley rats weighing 200-300 g were used. 1-2 mg of labelled sodium cholate was administered either orally or intraperitoneally in one or 3 ml of saline respectively. The rats were fed regular stock diet and water *ad lib.* Twenty-four or 48 hours after

* Supported in part by grant in aid from Dept. of HEW, Nat. Heart Inst.

† Department of Biochemistry, Baylor Univ. College of Medicine, Houston, Tex.

TABLE I. Calculation of Concentration of Cholic Acid and Its Metabolites in Blood of Rats Receiving Tritium Labelled Cholic Acid.

Animal	Cholic acid-T	Equilibration time, hr	cpm $\times 10^{-6}$, intestine	cpm $\times 10^{-6}$ in 100 ml blood	Blood "cholic acid," mg/100 ml*
1	1 mg i.p.	24	283	2.42	.128
2	1 " "	24	224	1.24	.083
3	2 " "	48	385	2.17	.085
4	2 " "	48	321	1.80	.084
5	1 mg p.o.	24	373	2.31	.093
6	2 " "	48	420	1.53	.055
7	2 " "	48	318	1.17	.055

* Assuming intestinal cholic acid and its metabolites to be 15 mg(12-15).

administration systemic blood was collected under light ether anesthesia by aortic puncture into a heparinized syringe. A measured volume was added dropwise into 20 volumes of 96% ethanol. Following thorough mixing the volume was diluted to 250 ml with ethanol. The small and large intestines were removed, cut into small pieces and extracted separately by refluxing 3 times for 2 hours in 80% ethanol. The extracts were filtered through glass wool into a volumetric flask and diluted to 1000 ml with ethanol. Radioactivity of all extracts was determined by counting in an infinitely thin layer on aluminium planchets in a Frieseke Hoepfner FH 51 methane gas-flow counter. Ten determinations were made on each sample.

The ethanol extract of the blood was filtered and evaporated. In most cases 5 mg each of cholic and glycocholic acids were added, and the residue extracted with butanol from an acidified water solution(11). After evaporation of the butanol the residue was subjected to reversed phase chromatography with 50% methanol as moving phase and 50% isooctanol-chloroform as stationary phase (phase system C(12)). A 4.5 g column was used. Two ml of the ethanol extract of the small intestine were treated in the same way.

Radioactivity of all the fractions collected from the column was determined and the proportion of different bile acids was calculated from the total radioactivity in the different bands. The activity retained on the column was negligible. The locations of glycocholic and cholic acids were determined by titrations of the fractions with 0.02 N NaOH in methanol.

Results and discussion. The tritiated cholic acid was given intraperitoneally to 4 rats; 2 were killed after 24 hours and 2 after 48 hours. Three rats received the acid orally; one of these was killed after 24 hours and the other 2 after 48 hours. Results of the isotope measurements of the blood and intestinal extracts are given in Table I. Between 5 and 11 ml of blood was obtained from each animal. The distribution of activity between small intestine and large intestine was approximately the same as that found in a previous investigation(13), *i.e.* about 10% was present in the large intestine. Table I shows a calculation of the concentration of labelled bile acids in the blood. The following assumptions have been made in this calculation: 1) The "pool" of cholic acid and its metabolites in the intestine is 15 mg and 2) The labelled bile acids in the rat are completely equilibrated with this unlabelled "pool." From the results of several independent investigations(12-15) it appears acceptable to assume a "cholic acid pool" of 15 mg. This figure has been multiplied by the ratio of radioactivity in 100 ml of blood to radioactivity in intestine, which gives a value for blood cholic acid and metabolites in mg %. The second assumption concerning equilibration of labelled bile acid with the unlabelled pool seems justified from earlier investigations(12). However, to exclude errors due to uneven distribution of the isotope, the labelled acid was given intraperitoneally and orally and was allowed to equilibrate for 24 hours or 48 hours. After intraperitoneal administration an uneven distribution of the isotope between blood and intestine would result in higher specific activity

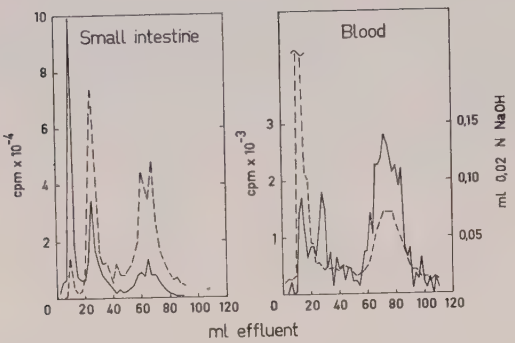


FIG. 1. Chromatograms of labelled compounds in small intestine and blood after administration of tritium labelled cholic acid. Solid line: radioactivity. Broken line: titration. Inactive glycocholic (20-30 ml) and cholic (50-80 ml) acids were added to small intestine, cholic acid (60-90 ml) was added to blood.

of blood bile acids than of intestinal bile acids. The reverse would occur after oral administration of labelled material. Under these circumstances intraperitoneal administration of labelled bile acid would result in higher blood bile acid values than those obtained after ingestion of this compound. In addition this difference would decrease with time. The blood bile acid levels obtained under the various conditions employed (Table I) support the assumption that a rapid equilibration of the labelled cholic acid takes place. The mean value found for cholic acid and its metabolites in whole blood was about 0.08 mg/100 ml. The value for serum cholic acid given by Friedman and Byers(7) is considerably higher but the specificity of the method used by these authors appears doubtful (2).

The nature of the labelled compounds in the blood was studied by reversed phase partition chromatography. Three main peaks of radioactivity were obtained which appeared in the effluent as taurocholic, glycocholic and cholic acids (Fig. 1). Since previous investigations (12) make it improbable that more than traces of other labelled acids could be present in these peaks, their identity was established only by chromatography together with inactive carrier compounds. Total activity in the 3 peaks was counted as 100% and the proportion between the peaks was calculated (Table II). The minute amounts of radioactivity retained in the columns represented incomplete elution of the compounds mentioned above and the presence of free deoxycholic acid could not be demonstrated.

A small percentage of free bile acids has previously been shown to be present in rat portal blood(8). Most of these acids represented metabolites formed in the cecum from the tritium labelled cholic acid administered, mainly deoxycholic acid. The surprisingly large percentage of free bile acids in combination with the absence of free deoxycholic acid in systemic blood made it improbable that these acids should have come from the cecum. It is highly improbable that some of the originally injected cholic acid should remain unconjugated after 24 hours and even more so after 48 hours. Therefore a chromatographic analysis was made of the labelled bile acids in the small intestine which contains the major part of the bile acid pool and that is the main source of portal bile acids. Contrary to previous results(12) fairly large amounts of free bile acids were found (Fig. 1 and Table II). Paper chromatography of small intestinal contents also showed spots corresponding to the labelled acids. The

TABLE II. Chromatographic Analysis of Labelled Bile Acids in Small Intestine and Blood of Rats Receiving Tritium Labelled Cholic Acid.

	Labelled bile acids in small intestine					Labelled bile acids in blood				
	Conjugated			Free	Ratio,	Conjugated			Free	Ratio,
Rat	Tau	Gly	Total		conj/free	Tau	Gly	Total		conj/free
1 i.p.	—	—	—	—	—	10	14	24	76	.3
2 "	—	—	—	—	—	21	26	47	53	.9
3 "	61	7	68	32	2.1	7	3	10	90	.1
4 "	45	20	65	35	1.9	20	6	26	74	.4
5 p.o.	80	10	90	10	9.0	57	11	68	32	2.1
6 "	82	9	91	9	10.1	42	13	55	45	1.2
7 "	40	32	72	28	2.6	12	18	30	70	.4

reason for this discrepancy is not known but it probably results from differences in the intestinal flora of the rats used in the two investigations. When a bile fistula is made on a rat from the strain used in this investigation only conjugated bile acids are excreted, mostly turocholic acid. This is in agreement with previous investigations.

The distribution of radioactivity between the 3 main bile acids found in the small intestine and blood is shown in Table II. The proportion of unconjugated bile acids is much higher in the blood than in the small intestine. The 2 rats (No. 5 and 6) having only small amounts of free bile acids in the small intestine also have a definitely lower percentage of free acids in the blood than the other animals. The results seem to indicate a preferential removal by the liver of the conjugated bile acids present in the portal blood.

Summary. Rats were given tritium labelled cholic acid intraperitoneally or orally and killed by exsanguination after 24 or 48 hours. The concentration of "total cholic acid" in whole blood was calculated from the distribution of isotope in intestine and blood and previous figures for intestinal "cholic acid." A value of about 0.08 mg/100 ml was found. Chromatographic analysis showed that more unconjugated cholic acid was present in the systemic blood than in the small intestine.

1. Sobotka, H., *Physiological Chemistry of the Bile,* Baltimore, Williams and Wilkins, 1937.

2. Mac Intyre, I., Wootton, I. D. P., *Ann. Rev. Biochem.,* 1960, v29, 635.

3. Rudman, D., Kendall, F. E., *J. Clin. Invest.,* 1957, v36, 530.

4. Osborn, E. C., Wootton, I. D. P., Da Silva, L. C., Sherlock, S., *Lancet,* 1959, vII, 1049.

5. Carey, J. B., Jr., *Science,* 1956, v123, 892.

6. ———, *J. Clin. Invest.,* 1958, v37, 1494.

7. Byers, S. O., Friedman, M., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 425.

8. Olivecrona, T., Sjövall, J., *Acta Physiol. Scand.,* 1959, v46, 284.

9. Wilzbach, E. E., *J. Am. Chem. Soc.,* 1957, v79, 1013.

10. Bergström, S., Lindstedt, S., *Acta Chem. Scand.,* 1957, v11, 1275.

11. Norman, A., *Acta Physiol. Scand.,* 1954, v32, 1.

12. Norman, A., Sjövall, J., *J. Biol. Chem.,* 1958, v233, 872.

13. Gustafsson, B., Norman, A., Sjövall, J., *Arch. Biochem. Biophys.,* 1960, v91, 93.

14. Portman, O. W., Murphy, P., *ibid.,* 1958, v76, 367.

15. Eriksson, S., *Acta Physiol. Scand.,* 1960, v48, 439.

Received March 20, 1961. P.S.E.B.M., 1961, v107.

Application of Physical Concepts to Dose-Response Relationships of Mixed Estrogens and Mixed Androgens. A Mathematical Analysis.* (26611)

HERBERT S. STRICKLER, ELEANOR L. SAIER AND ROBERT C. GRAUER

Department of Research in Endocrinology and Metabolism, William H. Singer Memorial Research Laboratory, Allegheny General Hospital, Pittsburgh, Pa.

The application of physical concepts to biological responsiveness with a view toward achieving a better understanding of biological phenomena has intrigued many workers in the field. We originally reported(1) on the biological responsiveness to topical application of varying mixtures of androsterone and dehydroepiandrosterone to chick combs. We recently reported(2) the results of injecting mixtures of estrone, estriol and estradiol 17β on biological response of the uteri of immature rats. Our results and discussions were based on conventional dose-response relationships. Our present aim was to see if our data would lend themselves to the concept of surface adsorption as set forth in the Langmuir(3) adsorption isotherm, especially since topical application involves direct ap-

* Supported in part by a grant-in-aid from U. S. Public Health Service.

plication of hormones to the target organ. Hitchcock(4) first pointed out the formal identity of the simplest Langmuir adsorption isotherm with an equation he derived from the law of mass action as applied to a reversible homogeneous reaction. Lineweaver and Burk(5), Levvy and Marsh(6) and more recently Stetten(7) have applied similar transformations to other biological data with good results.

For this presentation the formulations of Lineweaver and Burk(5), dealing with velocities of reaction, have been transformed into equations dealing with biological responses as follows:

$$\text{Response} = R = \frac{R_\infty C}{K_s + C} \quad \text{(I) or in reciprocal form } y = bx + a \text{ (II)}$$

where R_∞ is the response at infinite concentration C_∞, K_s is the dissociation constant of the surface-substrate complex, a is $1/R_\infty$, y is $1/R$, x is $1/C$ and b is K_s/R_∞. (*cf.* also Stetten(7)).

In applying the above concept of surface† adsorption of surface-substrate complexes to our problem of biological responsiveness we think of the target organ as containing one or more active sites which become covered with the hormone. As the concentration approaches infinity the response of the target organ levels off, approaching a maximum. If this is true, a straight line function can be obtained from this type of data by plotting $1/R$ against $1/C$ wherein R is response of the animal to the dose and C is dose or concentration. The equation of this line ($y = bx + a$) can then be determined by the method of least squares and with this information we can evaluate the original equation (I). The limiting factor R_∞ is defined as the reciprocal of the intercept, a, of this straight line and it can readily be seen from this that a large intercept infers a poor response. The constant b which is equal to K_s/R_∞ is defined by the slope of this line. The affinity constant (a/b) of the hormone for the surface may be interpreted as the reciprocal of K_s, the dissociation constant, which measures the ease of loosening the steroid from the enzyme.

When applying the above concepts to data of a biological nature one should always examine such data by plotting. This is to be done before an equation is derived by least squares since the lowest dose has the largest moment, and it can alter the fit of the equation markedly. For example, the fit of this reciprocal type of plot to the data for androsterone was excellent except at 1 gamma, which point was then discarded for the least squares calculation. The type of plot used by Stetten(7) (that usually employed for adsorption data) does spread the points out more evenly but in view of the behavior of the data in the terrace point region(Huggins (8)) we feel that our procedure is to be preferred for dose response data. Lineweaver and Burk(5) also favor the type of plot that we employ.

Results. Calculations using this formulation with our data for pure estrogens and androgens are presented in Table I. It is noted that estrone, which is less hydrophyllic than estradiol or estriol, has a much smaller affinity coefficient than either of the others. This concept, however, neglects biological interchange at the surface and assumes the same mode of action for all 3 estrogens. Note that b is equal to the dissociation constant K_s, measuring the ease of loosening the steroid from the enzyme, divided by the maximum biological power of the steroid, R_∞. This value for estradiol is markedly different from that of estrone and estriol (Table I). The basic dose response curves for the estrogens using this concept are shown in Fig. 1. Androsterone and dehydroepiandrosterone, on the other hand, do not show much difference in their affinity constants, as would be expected from the fact that they are both 3-OH, 17-ketosteroids. However, b differs markedly, being much smaller for the more active androsterone. The dose response curves for androsterone and dehydroepiandrosterone are presented in Fig. 2. The remarkably good fit of these steroids to this concept is noteworthy since the method of

† In this paper the term "surface" means the net system contributing to the observed (macro) effect; only diffusion into cells and enzyme activity at a mitochondrial site may be involved, or several stages may be utilized.

TABLE I. Constants of Langmuir Adsorption Equations Calculated from Experimental Data on Estrone, Estradiol 17β, Estriol, Androsterone and Dehydroepiandrosterone.

Hormone	Slope b	Intercept a	a/b affinity constant	a/b (calc.)	Limits of response: 1/a (Stetten Q)	1/a + control	Highest observed value
E_1 Estrone	.0176	.0104	.59	—	96.1	117.6	103.5
E_2 Estradiol	.0019	.0078	4.11	—	128.2	149.7	94.1
E_3 Estriol	.0018	.0514	4.36	—	19.5	41.8	45.3
% by weight $E_1 + E_3$							
90 + 10	.0122	.0093	.76	.97	107.5	130.4	109.4
75 + 25	.0079	.0112	1.42	1.53	89.2	112.2	106.8
50 + 50	.0134	.0125	.93	2.47	80.0	102.5	100.2
25 + 75	.0153	.0186	1.22	3.42	53.5	76.0	101.7
10 + 90	.0120	.0282	2.35	3.99	35.5	58.0	63.0
% by weight $E_2 + E_3$							
1.64 + 98.36	.0099	.0375	3.78	4.36	26.7	51.6	58.4
4.76 + 95.24	.0114	.0137	1.20	4.35	73.0	96.0	45.3
13.04 + 86.96	.0037	.0149	4.03	4.33	67.1	89.1	85.6
% by weight $E_1 + E_2$							
99.45 + .55	.0453	−.0063	—	.61	—	—	—
98.36 + 1.64	.0415	−.0068	—	.65	—	—	—
95.24 + 4.76	.0247	−.0016	—	.76	—	—	—
86.76 + 13.04	.0083	+.0098	—	1.05	—	—	—
68.97 + 31.03	.0119	−.0102	—	1.68	—	—	—
Androsterone	3.525	.635	.180	—	1.57	1.85	1.71
Dehydroepiandrosterone	10.124	1.507	.149	—	.66	.93	.88
% by weight A + D							
75A + 25D	4.208	.881	.210	.172	1.13	1.39	1.45
60A + 40D	6.784	.827	.122	.168	1.21	1.58	1.48
50A + 50D	4.534	.770	.170	.164	1.30	1.60	1.70
40A + 60D	5.406	.911	.169	.161	1.10	1.42	1.43
25A + 75D	4.655	.835	.179	.157	1.20	1.50	1.54

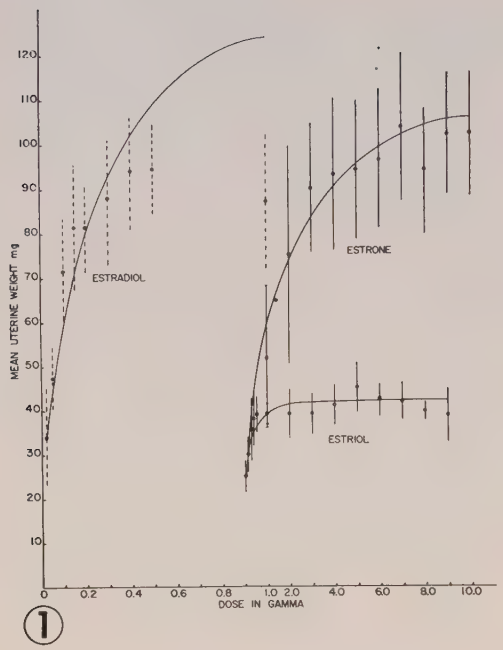
dosage presents a direct physiological pathway to reach the target organ, as contrasted with the parenteral mode of administration of the estrogens.

For the *mixtures,* equation (I) may again be plotted in the same fashion as was done for the pure hormones, *i.e.* 1/R versus 1/C or $y = bx + a$, where b and a, of course, do not have precisely the same meanings as for the single steroids. This was done for the various mixtures and the results have been summarized and included in Table I. Note that C is now the *total* concentration (*i.e.* the sum of the concentrations of the components). The proof for this procedure comes from a consideration of the Langmuir(3) relation for 2 gases competing for a surface.

Whence $\dfrac{1}{\text{Response}} = \dfrac{1}{R_\infty} + \dfrac{1}{R_\infty\left[\dfrac{B\rho + B'}{\rho + 1}\right]}$

$\dfrac{1}{C}$; $\rho = \dfrac{C_1}{C_2}$; $y = a + bx$ where R_∞ is maximum response for the particular mixture of estrogens, etc. in constant ratio ρ, C is total concentration and B and B′ are constants. Hence, a/b is $[(B\rho + B')/(\rho + 1)]$. On evaluating the constants B and B′, a/b becomes $\dfrac{a_1\rho/b_1 + a_2/b_2}{\rho + 1}$ (III). Hence, the affinity constant $K = a/b$ may again be calculated for each mixture from the constants of the responses to the pure hormones. Comparisons of such calculated and observed ratios are also given in Table I.

Limits of response have been calculated, for pure compounds and all mixtures, directly from the equation (I) and compared to observed values. The results in most all cases are quite good. The fit of the equation (I) to the data for pure estradiol 17β (Table I) is not as good, however, as desired at the limits of responsiveness. To fit the data used in computation of the effect of mixtures, it was necessary to favor the dose range before



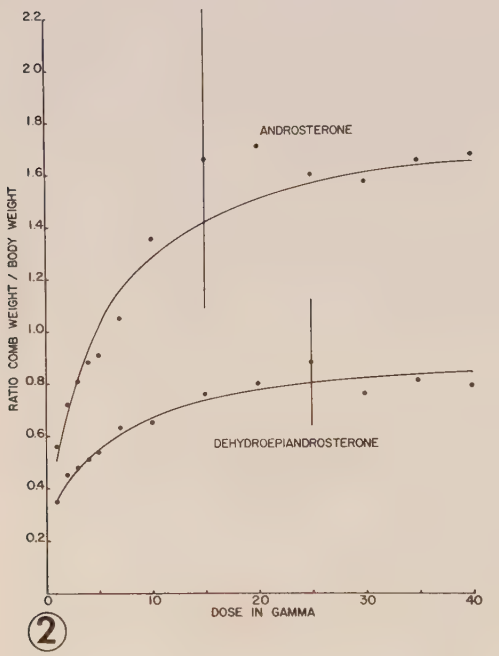


FIG. 1. Basic dose response data for estradiol 17β, estrone and estriol fitted by Langmuir adsorption concept (equation I) together with standard deviations (parenteral administration).

FIG. 2. Basic dose response data for androsterone and dehydroepiandrosterone fitted by Langmuir adsorption concept (equation I) together with the maximum standard deviation for each (topical application).

the threshold region. There is some indication in the original data(1,2) of a decreased response at the highest dosages. This, of course, is not predicted in the present theoretical work, which yields maximum response only at infinite dose.

To make further comparisons additive responses were calculated for each constituent of the dose in the mixture from the appropriate equation for the pure estrogen involved (Table I). These values were added and the sums then compared with the smoothed values obtained from the equations derived from the experimental data for each mixture (Table I). The results of these evaluations with each of the mixtures are discussed separately as follows:

Estrone-Estriol. Additivity was apparent through the entire dose range in the mixtures 90%-10% and 75%-25% but fell off at 8γ in 50%-50%, 7γ in 25%-75% and at 6γ in the 10%-90% mixture. Comparisons were made by taking into consideration the standard deviation for each dose response of the mixture.

Example: In the mixture 10% estrone-90% estriol(2) where the total dose was $C = 1.5\ \gamma$ ($C_1 = 0.15\ \gamma$ estrone + $C_2 = 1.35\ \gamma$ estriol) the actual observed response was 48.6 ± 5.6 mg. The calculated response was as follows: $\frac{1}{R}$ for estrone $= y_1 = .0176 \left(\frac{1}{C_1}\right) + .0104 = .0176 \times 6.667 + .0104 = 7.8$. $\frac{1}{R}$ for estriol $= y_2 = .0118 \left(\frac{1}{C_2}\right) + .0514 = .0118 \times .7407 + .0514 = 16.6$. $7.8 + 16.6 + 23$ (control) $= 47.4$ mg calculated additive uterine weight. The value for this point from the smoothing of the experimental data with the Langmuir type of plot is 50.5 mg and the experimental standard deviation is $\pm$ 5.6 mg indicating additivity in all respects. Above the region of additivity, the calculated additive responses are significantly higher than the observed responses, indicating a damping of the response of estrone by the highly hydrophilic estriol. The affinity constants for the mixtures rise with increasing estriol content and the

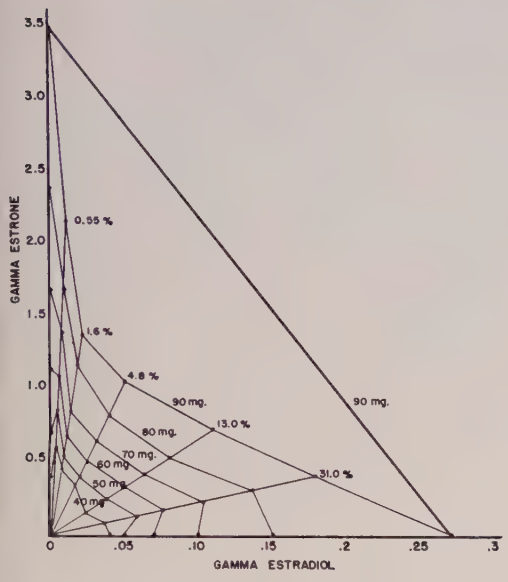


FIG. 3. Dose response curves for mixtures of estrone and estradiol plotted as an isobologram (Loewe(9)) showing synergism.

intercept value approached or was even less than either of the pure steroids (Table I). Agreement of a/b (affinity constant) calculated from equation III with those from the smooth (Langmuir) experimental data is only fair (columns 4 and 5, Table I). This may indicate that there is more than one active surface.

Estradiol-Estriol. In the combination of these 2 estrogens, additivity is found through the dose range 1.525 γ for mixtures 98.4% + 1.6% whereas in the mixture containing 94.2% + 4.8% additivity extends to the dose range of 3.675 γ. However, it falls again at 1.4376 γ dose in the mixture 86.96% + 13.04%. It is noted that a suggestion of synergism is seen in the response to dose in the 95.2% + 4.8% mixture. Good agreement of calculated and observed affinity constants (a/b) is observed in 2 out of the 3 mixtures. The mixture which is synergistic gives a sharply different affinity constant.

Estrone-Estradiol. This particular group of mixtures does not lend itself to the concept of surface adsorption. Inspection of Table I shows immediately the negative intercept. Additive responses were calculated, however, using the equation for the pure compounds and compared with the individual experimental points and their standard deviations. The results are as follows:

99.45% + 0.55% additivity over the entire dose range within limits of experimental error.

98.36% + 1.64% additivity to dose of 6.86 γ

95.24% + 4.76% additivity to dose of 3.675 γ

86.96% + 13.04% additivity to dose of 1.4376 γ

68.97% + 31.03% additivity to dose of 0.7250 γ

These mixtures do seem, however, to show evidence of synergism. We have, therefore, used a method of plotting as suggested by Loewe(9) known as an isobologram‡ (Fig. 3). The isobologram was constructed from data read off smoothed curves which were drawn empirically since the Langmuir type of equation did not fit this set of data. Note in Fig. 3 that straight lines drawn between points of equal response for the pure estrogens are found *above* the majority of the experimental points.

Androgens. The calculated affinity coefficients (a/b) for mixtures of androsterone and dehydroepiandrosterone agree fairly well with the experimental values. The slopes (b) and intercepts (a) hover much closer to pure androsterone than dehydroepiandrosterone (cf Table I). It is to be stressed that the hormonal mixtures of androgens were applied directly to the target organ, the chick comb. Hence, the complications inherent in remote injection, by which the estrogens were administered, are mainly avoided. It is true that diffusion into the circulation from

‡ Isobolograms illustrate changes which properties of mixtures undergo, if quantity and relative proportions of the mixture components are continuously changed. The rays show points of the same relative proportions, the isoboles are the connecting lines of those mix points which show a definite biological response (in this case) to the same degree. Synergism is indicated when a line drawn diagonally between 2 points of the coordinate axes (pure estrogens) giving the same response, has all points of the isobole under this line.

TABLE II. Predicted Uterine Response.

Ratio, estrone/estriol	.5 γ estrone γ estriol	.5 γ estrone Uterine wt (mg)	1.5 γ estrone γ estriol	1.5 γ estrone Uterine wt (mg)
100: 0	0	44.9	0	68.2
90:10	.05	54.3	.17	82.8
75:25	.16	65.6	.50	89.5
50:50	.50	61.1	1.50	82.1
25:75	1.50	60.5	4.50	70.1
10:90	4.50	55.6	13.50	57.4

the chick comb undoubtedly occurred and also back diffusion of metabolic products (from liver, etc.) but the effect of these would be expected to be minor in comparison with the direct impact of the hormone.

Discussion. The equation presented in the previous section now provides a "tool" which can be used in various ways to examine trends in data obtained from the actions of mixed estrogens and/or androgens. These equations provide a continuity not generally provided by experimental data and therefore it becomes possible to calculate responses with a fixed amount of one component and variable amounts of another by using the various ratios determined experimentally.

To illustrate this, we have taken our data for estrone and estriol and thus can predict what may occur if one constituent is kept constant and the other varied (Table II). When these data are plotted the pattern is very similar in the low dose range to the recent work of Huggins and Jensen(8) with estrone and 16 epi-estriol as illustrated in their Fig. 4.

These calculations based on surface concepts naturally breed speculations as to such effects in other systems. Dorfman *et al.*(10) have recently noted that when the 17α hydrogen is replaced by an alkyl group (17α methyl) in 2αmethyl-17-β-hydroxy-androstane 3-one, the anti-estrogenic effect is increased 25 times. We postulated that this may be due to the increased screening action thus afforded against estrogen attachment at an active surface.

Summary. 1. An equation, Response $= \frac{R_\infty C}{K_s + C}$, based on the concept of surface adsorption as set forth in the Langmuir adsorption isotherm has been derived as an aid in understanding biological response to steroids. 2. The equations provide a tool by which theoretical predictions can be correlated with experimental data. 3. The Langmuir concept is given added support since the equation is valid whether the steroid is applied topically as in the androgens or parenterally as in the estrogens.

1. Grauer, R. C., Starkey, W. F., Saier, E. L., *Endocrinology,* 1948, v42, 141.

2. Grauer, R. C., Saier, E. L., Strickler, H. S., Cutuly, E., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 308.

3. Langmuir, I., Phys. Rev., 1913, v2, 331 and 1915, v6, 79.

4. Hitchcock, D. L., *J. Am. Chem. Soc.,* 1926, v48, 2870.

5. Lineweaver, H., Burk, D., *ibid.,* 1934, v56, 658.

6. Levvy, G. A., *Science,* 1954, v119, 337.

7. Stetten, DeWitt, Jr., *ibid.,* 1956, v124, 365.

8. Huggins, C., Jensen, E. V., *J. Exp. Med.,* 1955, v102, 335.

9. Loewe, W. S., *Klin. Wochenschrift,* 1927, v6, 1078.

10. Dorfman, R. I., Kincl, F. A., Ringold, H. J., *Endocrinology,* 1961, v68, 17.

Received March 20, 1961. P.S.E.B.M., 1961, v107.

Biological Profile of Various Growth Hormone Preparations. (26612)

Elliott J. Collins and V. F. Baker
Department of Endocrinology, The Upjohn Co., Kalamazoo, Mich.

Biological activity of primate, beef and whale pituitary growth hormone as evidenced by body wt gain and the tibia assay was investigated by Li, *et al.*(1). These preparations were observed to have almost identical growth-promoting potencies in the tibia test, but were not comparable on the basis of body weight gain; thus the interest to investigate the activities of these preparations in stimulating incorporation of radio-sulfate in costal cartilage of hypophysectomized rats.

Methods. Female rats, Holtzman strain, were hypophysectomized* at 21 ± 2 days of age, housed at an environmental temperature of 78 ± 2°F and fed Purina laboratory chow *ad lib.* Two weeks after hypophysectomy, animals weighing between 65-80 g were used for experimentation, 8 animals/dosage group.

1. *Dose-response study.* Animals were given a single intraperitoneal (I.P.) injection of the test preparations. Hormone preparations were dissolved in isotonic saline so that each ml contained the desired dosage. Eight hours later, 20 μc S^{35} as sodium sulfate dissolved in 1 ml isotonic saline (without added carrier) was administered I.P. Sixteen hours later (24 hrs after hormone injection) the animals were sacrificed and the VII costal cartilage removed. This procedure has been shown to be a reliable index for growth hormone activity(2,3). 2. *Duration of response study.* Animals were given a single I.P. injection of 12 μg test preparation. At various time intervals over a 48-hr period, animals were sacrificed and VII costal cartilage removed. *Four hours prior to sacrifice,* 10 μc S^{35} was administered I.P.

Cartilage was cleaned of adhering tissue, weighed, and treated by the procedure previously described(2). Radio-sulfate was counted in a gas-flow end window counter and results reported as CPM/mg tissue, wet weight.

Results. 1. *Dose-response study.* Growth hormone preparations isolated from human, monkey, and beef pituitary glands appear to have equal activity in promoting radio-sulfate incorporation in costal cartilage of hypophysectomized rats. Material obtained from whale, pig, and sheep pituitaries demonstrated equal activity but approximately 50% of that observed for human, monkey, or beef preparations (Table I). Calculated potencies presented in Table IV are based on 3-12 μg doses. With the exception of human and pig preparations, the response appeared to plateau at doses above 12 μg.

When these 6 preparations were compared for their ability to increase width of epiphyseal plate, human, monkey, and beef materials were observed to have almost identical activity (Table III). Pig and whale preparations demonstrated 100% and 50% more activity, respectively, whereas the sheep preparation was least active. 2. *Duration of response study.* Activity of these preparations, based upon duration of action of a single 12 μg dose, was studied. Radio-sulfate incorporation was increased relative to un-

TABLE I. Effect of Various Growth Hormone Preparations on Radio-Sulfate Incorporation of Costal Cartilage.

Preparation	Control	cpm/mg tissue ± S.E.					
		Human	Sheep	Pig	Whale	Monkey	Beef
Control	29.0 ± 1.9						
3 μg GH		43.1 ± 4.0	37.0 ± 3.1	33.5 ± 2.9	35.5 ± 1.3	41.3 ± 3.2	40.0 ± 3.3
6 μg "		50.2 ± 3.6	37.4 ± 2.8	38.3 ± 3.0	37.1 ± 2.0	45.7 ± 6.1	47.0 ± 2.3
12 μg "		53.0 ± 5.7	49.1 ± 4.4	48.7 ± 4.1	50.7 ± 4.2	56.7 ± 5.1	59.4 ± 8.2
24 μg "		71.5 ± 4.9	45.8 ± 4.9	66.2 ± 9.6	49.8 ± 4.5	55.7 ± 9.0	59.8 ± 9.9

* Hormone Laboratories, Chicago, Ill.

TABLE II. Duration of Activity of Single Dose of Various Growth Hormone Preparations on Radio-Sulfate Incorporation of Costal Cartilage.

Hr post-hormone	cpm/mg tissue ± S.E.						
	Control	Human	Sheep	Pig	Whale	Monkey	Beef
24	10.8 ± .4	15.1 ± 2.6	17.1 ± 3.6	13.4 ± 1.1	14.7 ± 2.2	11.0 ± 2.2	13.9 ± 3.1
28	9.7 ± 1.0	16.7 ± 2.0	12.7 ± 1.0	18.6 ± 1.4	16.1 ± 2.9	16.7 ± 1.1	13.6 ± 2.4
32	9.0 ± .2	17.1 ± 1.8	14.4 ± 1.5	12.0 ± 1.2	18.1 ± 1.9	13.2 ± 1.5	16.4 ± 1.1
36	6.2 ± .5	10.0 ± 1.1	8.8 ± 1.2	12.2 ± 1.8	12.4 ± 1.1	8.3 ± .7	8.2 ± 1.2
40	5.5 ± .6	10.8 ± 1.9	8.9 ± .6	8.4 ± .7	9.8 ± 1.9	8.2 ± .7	12.0 ± 1.4
48	7.0 ± 2.2	8.7 ± .9	8.4 ± 1.0	8.9 ± 1.2	11.0 ± 1.1	9.2 ± 1.1	8.3 ± .9

TABLE III. Assay of Various Purified Growth Hormones by the Tibia Test.*

Growth hormone	Response† Total dose in 4 days			Slope	Index of precision
	20 μg	60 μg	120 μg		
Human	213 ± 2 (8)	235 ± 2 (8)	256 ± 2 (6)	52.9	.129
Monkey	210 ± 4 (4)	242 ± 5 (6)	261 ± 3 (5)	65.7	.147
Beef	206 ± 3 (5)	242 ± 1 (4)	248 ± 6 (4)	56.3	.095
Pig	226 ± 4 (7)	243 ± 4 (5)‡	279 ± 4 (6)§	60.8	.168
Whale	220 ± 4 (5)	250 ± 2 (5)	268 ± 4 (5)	62.6	.130
Sheep	209 ± 2 (4)	231 ± 3 (5)	249 ± 4 (6)	52.6	.157

* Unpublished results (private communication from C. H. Li).
† In terms of mean tibia width ± stand. error. No. of rats in parentheses.
‡ 40 μg total dose.
§ 100 " " " .

treated animals during the first 24 hours with all preparations and remained elevated during the 48-hour experimental period (Table II). Increased uptake of isotope continued through the 32nd hour and was observed in all groups. Peak response varied from 24 hours (sheep) to 32 hours (whale). These observations of prolonged effect of growth hormone compared with the relatively short-lived effects of ACTH are of significant interest and should provide useful methods for study of metabolism and mechanism of action of growth hormone.

Discussion. Using the tibia assay and body weight gain, Li *et al.*(1) found that whale and beef growth hormone had greater effect on body weight gain than did human or monkey hormones, but the 4 preparations appeared to have almost identical growth-promoting activity in stimulating epiphyseal tissue.

TABLE IV. Comparative Potencies of Growth Hormone Derived from Several Mammalian Species.*

Species	S^{35} assay	Tibia test
Human	1.00	1.00
Monkey	.920	1.12
Beef	1.054	.90
Pig	.438	2.13
Whale	.477	1.54
Sheep	.481	.83

* Calculations based on data presented in Tables I and III.

We have investigated these hormone preparations for their ability to stimulate incorporation of radio-sulfate into costal cartilage. It was observed that human, beef, and monkey preparations were nearly equally active, and the calculated potencies derived from the 24-hr radio-sulfate assay and 4-day tibia assay were not significantly different.

Similar correlations were not obtained with whale and pig preparations. Recent data from Dr. Li's laboratory (Table III) show whale and pig growth-hormone preparations to possess greater activity in the tibia assay than human, beef, and monkey materials. In the 24-hour radio-sulfate uptake assay, whale and pig material were observed to have approximately 50% the activity of human, monkey, or beef growth hormone. If modification of the growth hormone molecule to

a more active form, by rat tissues, is required, rate of utilization would be relatively slower and a 24-hour test might well be too short a period for quantitative comparison.

It is interesting to note that, following a single injection, whale and pig growth hormones had the same duration of activity and magnitude of response as did human, monkey, and beef preparations in the time intervals studied. This could be a further indication that the difference between the 2 assays is a function of time dependent upon modification of the growth hormone molecule and/or slower rate of utilization of whale and pig material.

The fact that the 2 assays for growth-promoting activity give quantitatively different results suggests that the short-term assay is of value for qualitative measurement and that quantitative comparisons require a longer test period.

Summary. Growth hormone isolated from 6 different species was compared for its ability to increase width of epiphyseal plate and to stimulate radio-sulfate incorporation by costal cartilage. Human, beef, monkey, and sheep preparations manifested almost identical effects in the 2 assays. Whale and pig hormones appeared to elicit greater effect in the tibia test. Whale growth hormone was observed to have greater duration of activity to stimulate radio-sulfate incorporation than other preparations studied. Short-term radio-sulfate assay appears to be of value for qualitative detection of growth-promoting activity.

The authors acknowledge the cooperation, suggestions, and growth hormone materials supplied by Dr. C. H. Li.

1. Li, C. H., Paphoff, H., Johnson, C. W., Proc. Soc. Exp. Biol. and Med., 1959, v100, 44.
2. Collins, E. J., Lyster, S. C., Carpenter, O. S., *Acta Endoc.*, 1961, v36, 51.
3. Collins, E. J., Baker, V. F., *ibid.*, 1961, v37, 176.

Received March 27, 1961. P.S.E.B.M., 1961, v107.

Determination of "Heparinoid" Substances in Urine with a Dye-Binding Technic.* (26613)

Louis Rosenfeld,† Harold Neuhof and A. L. Mestel
(Introduced by David M. Spain)
Dept. of Laboratories and the Clinical Services, Beth-El Hospital, Brooklyn, N. Y.

Although crystalline heparin of known chemical composition and with reproducible physiological properties is available for clinical and laboratory study, the basis of its action in blood clotting and other metabolic activities has not been completely elucidated. Complicated chemical methods have revealed very small amounts of heparin in the circulating blood, too minute, however, for quantitative determination.

Early investigators were not in agreement as to the fate of heparin, whether endogenous or administered. Howell and McDonald(1) were first to report the presence of heparin in urine of dogs following injection. Copley and Schnedorf(2) using a relatively simple dye-precipitation technic, found that from 9.9-35.6% of injected heparin was excreted in urine of dogs within 110 minutes. There was no activity before administration. Jaques(3), using a metachromatic technic, and Astrup(4), with an anticoagulant procedure, reported daily excretion in urine of man of amounts up to 0.7 mg heparin without prior injection. When injected, excretions of 2-15%(3) and 1.6-12%(4) were obtained.

The excreted material is not unchanged heparin, as seen in the disparity between metachromatic activity and the lesser clot-

* Supported by a grant from the Dr. Marcus K. Goldsmith and Clara Harris Goldsmith Medical Research Foundation, Inc.

† Present address: Dept. of Pathology, University Hospital, N.Y.U.-Bellevue Medical Center, N. Y., N. Y.

inhibiting ability of the isolated substance (3-7). *In vivo,* these and other related substances have been characterized as "heparinoid"(8).

The method was developed from the procedures of Copley(2,9), modified and made quantitatively more accurate and reproducible. Following centrifugation of a mixture of heparin and toluidine blue, an inverse linear relationship was found to exist between concentration of heparin and optical density of the unreacted dye supernatant.

Indirect determination of heparin or other dye-binding substances ("heparinoids") by separation in the insoluble complex with toluidine blue appeared to be a logical approach.

Methods and materials. One milliliter of toluidine blue‡ solution, 0.0125%, in 0.067 molar phosphate buffer, pH 7.2, is added to 0.5 ml of centrifuged test urine in a microcuvette (10×75 mm). The solution is mixed by tapping and allowed to stand for 15 minutes at room temperature. The dye and "heparinoids" react to form an insoluble purple complex which settles out during 5 minutes of centrifugation. The supernatant is not decanted and its per cent transmittance or optical density is read in a spectrophotometer at 700 mμ against a blank of urine and buffer. Units of reacting substance are interpolated from a standard calibration graph and are reported as total units of "heparinoids" excreted per hour. The standard graph is prepared by reacting 1.0 ml toluidine blue solution with 0.5 ml volumes of phosphate buffer containing 1 to 5 units of heparin§ respectively. The standard graph should be checked for each new preparation of diluted dye solution, since small fluctuations occasionally occur. The heparin dilutions are stable for several months, refrigerated.

‡ Supplied by Abbott Laboratories, North Chicago, Ill. A stock solution of 0.200% powdered dye in the phosphate buffer was prepared.

§ Supplied by Lederle Laboratories, Pearl River, N. Y.;
100 mg/ml
100 units/mg

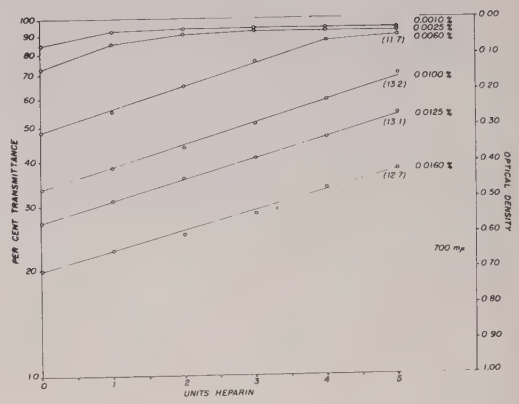


FIG. 1. Relationship between per cent transmittance (optical density) and concentration of heparin at 700 mμ for several concentrations of toluidine blue. The gamma of dye decolorized by one unit heparin is indicated in parenthesis. The value at 0.0060% was determined for the major linear portion of graph.

The subjects tested were normal individuals who were in no way restricted during the test. A 2-hour urine specimen was collected just prior to administration of 25 mg (2500 units) of heparin. Injection was made slowly, admixing with blood, with a tuberculin syringe. The urine voided during the 2-hour period following injection was collected and both specimens were analyzed. The increase of dye-binding substances in the second specimen over that in the first was calculated and reported as per cent of injected heparin.

Results. Utilization of 700 mμ and 0.0125% toluidine blue (Fig. 1) best meets the requirements of sensitivity and linearity of response over the range encountered for the test. Identical graphs were obtained for freshly prepared reaction mixtures at pH 5.5, 7.2, and 8.3, whether in distilled water or phosphate buffer at 0.067 or 0.133 molar. The dye at pH 5.5 and 7.2 is stable, but at pH 8.3 there is considerable fading of color within a week, necessitating constant restandardization. The reaction of most freshly voided urines is slightly acidic and after addition of the pH 7.2 buffered dye solution the pH of the mixture falls at about 6.7 ± 0.5. Under these conditions duplicate analyses are in excellent agreement and reproducible results are obtained with urines

TABLE I. Enhancing Effect of Urine on Reaction between Heparin and Toluidine Blue.

			"Heparinoid" units†			
			Before dialysis		After dialysis	
	Urine, ml	Heparin, ml*	Theoretical	Analytical	Theoretical	Analytical
A.	.25	1.75	4.41	4.73	4.33	4.55
	.50	1.50	3.81	4.35	3.86	4.00
	1.00	1.00	2.62	3.17	2.71	2.82
	1.50	.50	1.43	1.80	1.57	1.62
	1.75	.25	.84	.90	1.00	1.04
	2.00	.00	—	.24	—	.40
B.	.25	1.75	4.56	4.94	4.56	4.58
	.50	1.50	4.11	4.63	4.10	4.10
	1.00	1.00	3.22	3.75	3.20	3.27
	1.50	.50	2.33	2.60	2.30	2.31
	1.75	.25	1.89	1.95	1.83	1.85
	2.00	.00	—	1.44	—	1.40

* 10 units/ml.

† Per ½ ml test mixture.

stored at refrigerator temperature for periods up to 3 weeks.

The amount of dye (gamma) reacting with one unit of heparin at different concentrations of toluidine blue is indicated in Fig. 1. These were determined by calculating decrease in optical density per unit heparin, and units of optical density per gamma of toluidine blue. One unit of heparin was found to react with (decolorize) 13 γ of dye. This agrees with the value of 15 γ reported by Copley and Whitney(10), and calculated by the present authors from the graph of MacIntosh(5).

The competitive action of proteins for heparin(11) in reaction with toluidine blue was studied because of the occasional presence of trace amounts of albumin in urine of some patients. The data obtained showed there is no interference with urinary "heparinoid" (or pure heparin) at serum protein concentrations up to 1.0% for "heparinoid" levels as high as 3.5 units per 0.5 ml test specimen. When present in larger amounts, the protein does prevent the heparin from reacting with the dye, and false low results are obtained. The toluidine blue test is thus of limited value for specimens with a proteinuria greater than +2, and the specimen must be diluted before analysis.

There is an enhancing substance present in urine, which is responsible for a marked though variable increment in recovery experiments. The results of 44 such experiments show the enhancement to vary from 9-43%. This effect is present even in urines containing no measurable dye-binding substance, and has been noted by Jaques(3). After prolonged dialysis of urine, mixtures with heparin yield analytical results in agreement with theoretical values. Table I presents recovery data before and after dialysis for 2 urines of different "heparinoid" content. That the enhancing material has an effect only on added heparin in mixtures with urine, and not on excreted "heparinoids" is shown by the fact that results for dye-binding substance are the same before and after dialysis.

The enhancing factor is not related to urinary pigment since dark and light colored urines of the same content of dye-binding substance were equally effective. When aliquots of several urine specimens are mixed, there is no enhancing effect noted. Thoretical and analytical results are in agreement.

In Table II are found mean hourly excretions of dye-binding substances in the urine of normal subjects. Specimens were collected for varying intervals of time (2-10

TABLE II. Excretion of "Heparinoids" in Urine of Normal Subjects.

Subject	Age	No. of subjects	No. of specimens	"Heparinoid" units/hr
Males	20-53 yr	24	139	29 ± 11*
Females	18-52 "	31	95	16 ± 13
Children	2-12 "	25	40	80 ± 31
Infants	1- 8 days	18	18	16 ± 14

* Mean ± S.D.

hours) during all parts of the day. There was no significant variation during any part of the day. When more than one specimen from an individual was tested, the values were averaged before being included in determination of the mean. One premature infant (male) was studied weekly for 6 weeks. His values were the same as that of the full-term infants. The difference in values for men, women, and children (boys and girls) is significant. For the men and women, $t = 3.8$. Several specimens daily, representing from 16-24 hours, were collected from 2 adults for extended periods and analyzed. Daily averages were calculated, their mean determined and reported as units of "heparinoid". For the male, the mean of 13 days (42 specimens) was 34 units ± S.D. 10 units "heparnoid" per hour; for the female, the mean of 14 days (29 specimens) was 12 units ± S.D. 11 units "heparinoid" per hour. These values agree with the mean for their groups.

Following an intravenous test dose of 25 mg heparin, 14.5% ± S.D. 4.1% was excreted in a group of 27 normal men and women ranging in age from 16-72 years. There was no significant difference between the sexes in this respect.

The results of a study of 31 women making weekly visits to the prenatal clinic during the second half of their pregnancy are presented in Table III. A substantial increase in excretion of dye-binding substance is noted as early as the 6th month and probably exists earlier. Peak levels are reached during the middle of the third trimester. These increments over normal approximate those following the test dose. Twelve women were followed daily during their 7 days of post-partum care and the mean of 55 specimens indicates a return to normal levels.

Discussion. Analyses of urine specimens before injection of the standard dose revealed the presence of measurable amounts of reacting material. Substantial quantities of dye-binding substance were found in the urine of children (50-120 units "heparinoid" per hour). In infants small amounts approximating those found in women are excreted. The urine of women (0-30 units "heparinoid" per hour) contains less than that of men (20-40 units "heparinoid" per hour). There is no variation during the menstrual cycle. A similar significant difference in the urines of men, women and children has been reported by other investigators(12-14) using 2 entirely different analytical procedures for measurement of acid mucopolysaccharides. These compounds reacted with toluidine blue and when chromatographed on paper were identified chiefly as chondroitin sulfate.

TABLE III. Excretion of "Heparinoids" during Pregnancy.

Wk before parturition	Subjects tested	"Heparinoid" units/hr, mean
9–14	19	91
8	4	85
7	10	71
6	7	119
5	12	125
4	15	113
3	21	124
2	24	114
1	21	137
Daily, 1 wk after delivery	12	65

The great increase during pregnancy presumably reflects the overall change in body metabolism. Peak levels are reached in the middle of the third trimester, followed by a decrease in the first post-partum week. In a study of post-operative patients a pronounced increase in excretion of dye-binding substance occurred on the first or second day followed by a gradual and sometimes erratic subsidence to pre-operative levels.

Conclusions. 1. A quantitative method based on the reaction of toluidine blue with heparin and "heparinoids" is described. It reveals a range of 9-24% output in the urine of injected heparin in normal individuals following a standard test dose of 25 mg of heparin. 2. The presence of dye-binding substances in varying amounts in the urine of infants, children, men, women, and during pregnancy, without an injection of heparin, suggests the existence of a "heparinoid" metabolism.

1. Howell, W. H., McDonald, C. H., *Bull. Johns Hopkins Hosp.*, 1930, v46, 365.

2. Copley, A. L., Schnedorf, J. G., *Am. J. Physiol.*, 1941, v133, 562.
3. Jaques, L. B., Napke, E., Levy, S. W., *Circ. Res.*, 1953, v1, 321.
4. Astrup, P., *Acta Pharmacol. et Toxicol.*, 1947, v3, 165.
5. MacIntosh, F. C., *Biochem. J.*, 1941, v35, 776.
6. Bassiouni, M., *J. Clin. Path.*, 1954, v7, 330.
7. Marbet, R., Winterstein, A., *Helv. Physiol. et Pharmacol. Acta*, 1951, v9, 24. *C. A.*, 1951, v45, 7179.
8. Thomas, W. A., Olwin, J. H., *Angiology*, 1958, v9, 279.
9. Copley, A. L., Whitney, D. V., III, *J. Lab. & Clin. Med.*, 1943, v28, 762.
10. ———, *ibid.*, 1944, v29, 117.
11. Jaques, L. B., *Biochem. J.*, 1943, v37, 189.
12. Kerby, G. P., *J. Clin. Invest.*, 1954, v33, 1168.
13. Di Ferrante, N., Rich, C., *J. Lab. & Clin. Med.*, 1956, v48, 491.
14. Di Ferrante, N., *J. Biol. Chem.*, 1956, v220, 303.

Received March 28, 1961. P.S.E.B.M., 1961, v107.

A Serological Reaction Associated with Sarcoidosis.* (26614)

JOHN S. CHAPMAN (Introduced by Donald W. Seldin)
Department of Internal Medicine and William Buchanan Laboratory, University of Texas Southwestern Medical School, Dallas, Texas

At present the diagnosis of sarcoidosis is based on clinical grounds, supported by either a compatible histological pattern or a positive Kveim test(1). Since many other agents are known to produce the so-called "hard tubercle"(2) and since a potent and reliable Kveim antigen is difficult to obtain, final diagnosis is often accomplished by exclusion.

When potent Kveim antigen is available positive reactions are obtained in about 75% of cases(3). If another positive test possessing a fair degree of consistency can be found, the uncertainties resulting from diagnosis by exclusion may be somewhat reduced. Since the reported distribution of non-photochromogenic anonymous mycobacteria resembles somewhat the distribution of sarcoidosis as described in Veterans Administration studies, it seemed worth while to study sera of patients with sarcoidosis against antigens of various types of anonymous mycobacteria. If antibodies should be found they might also suggest clues as to the etiology of sarcoidosis.

The following studies were undertaken to see if serum of patients with sarcoidosis possessed antibodies detectable by diffusion against any of a number of mycobacterial and fungal antigens. Diffusion in agar was chosen as a method since Parlett and Youmans(4) have found the technic useful in mycobacterial study, while Heiner(5) has used diffusion in the study of histoplasmosis, and experience in this laboratory indicates its usefulness in other fungus infections.

Materials and methods. Sera from 32 patients with sarcodosis as determined by usual clinical criteria supported by Kveim test, biopsy or both were subjected to double diffusion in agar gel. These sera were distributed geographically as follows: Virginia 8, Wisconsin 5, Oklahoma 5, Louisiana 7, and Texas 7.

Diffusion was carried out in Petri dishes, using wells and distances as described by Crowle for slide technic(6). A 1% solution of agar was poured in the dish and allowed to harden. A second pour of 1½% ordinary bacto-agar in physiological saline was then layered on the first pour, and as it first began to harden a tooled die was placed on the surface, forming wells of a constant size and a fixed distance.

The central well was filled with undiluted serum and the peripheral wells with various antigens. In a few instances when the first

* This study was made possible through a grant-in-aid from the Dallas Tuberculosis Assn.

diffusion was negative a second diffusion was carried out with the serum diluted 1:1 and 1:2 with 0.7% NaCl. All sera were studied against the antigens described below.

Mycobacterial antigens were prepared by inoculating Sauton's liquid media from fresh subcultures of strains P-1 and P-8 of Group I; P-6 and P-15 of Group II; and P-2 and P-17 of Group III. The original subcultures of these strains were prepared by Dr. Ernest W. Runyon from his strain collection and were members of the "packs" Runyon circulated for study and identification by numerous laboratories. The further technic of preparation of antigens has been described elsewhere. After the Old Tuberculin-like antigens had been concentrated by flash evaporation, the lipids were extracted with ether and discarded, and the remaining material used as antigen.

Old Tuberculin derived from H37Rv was obtained from the Texas State Dept. of Health and was extracted with ether in the same way.

Blastomyces antigens were prepared in this laboratory from an organism isolated from lung and pleura of a local patient. Antigen for *Coccidioides immitis* was furnished by Dr. C. E. Smith, Univ. of California, and is the material employed by him in complement-fixation studies. Histoplasma antigens were furnished by Furcolow and also a commercial product from Eli Lilly & Co. Antigens for Cryptococcus were produced in this laboratory from a Sabouraud broth culture of organisms isolated from a resected lesion of the lung.

When the wells were filled, the Petri dishes were covered and placed in the desiccator for 1 hour, then they were placed in an incubator at 37° for 24 hours in the case of the fungus diffusion, and 48 hours for mycobacterial diffusion. Upon removal of Petri dishes the results were read with the aid of oblique light and a hand lens when necessary (Fig. 1).

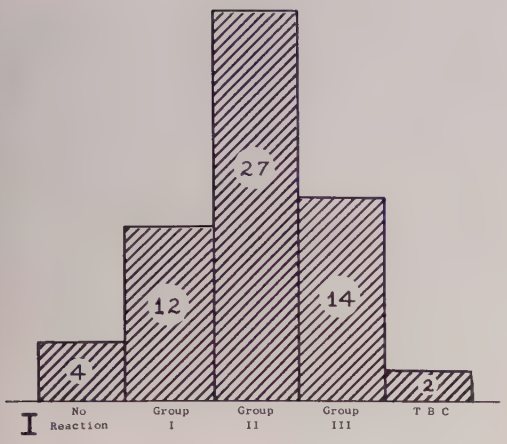
Results. Twenty-eight of 32 sera produced zones of precipitation against antigens of anonymous mycobacteria (Charts I, II). All of these diffusions resulted in 2 bands, while a few sera produced 3 or 4. The greatest number of reactions, as well as the most numerous bands, occurred against Group II antigens, while lesser numbers of reactions occurred against Groups I and III. Chart II, which presents reactions by combinations, shows that many sera produced precipitation against antigens of more than one group. The serum of a patient in Virginia produced the only reaction against any of the fungal antigens, and only 2 sera reacted with H37Rv antigen.

FIG. 1. Agar diffusion pattern obtained with serum of sarcoidosis patient. Antigens clockwise from top:
Photochromogen (P-1, P-8)
Scotochromogen (P-6, P-15)
Non-chromogen (P-2, P-17)
H37Rv antigen
P-6 alone
P-1 "

Three local patients are of particular interest in that sera obtained from each of these at the time they originally presented for diagnosis were uniformly negative. As these patients were followed and roentgenographic improvement was obvious the sera of all 3 produced multiple bands in from 5 to 9 months after the original study.

Discussion. Group II anonymous mycobacteria, which are regarded as the least pathogenic for man, elicited the most numerous and extensive reactions. In general the distribution of antibody reactions to antigens of Groups I and III corresponds to the reported geographic predominance of the groups. However, multiple antigen reactions may with equal probability represent identity of antigen components, or separate mycobacterial experiences.

Control sera have been obtained from medical students in the first 2 years, from a



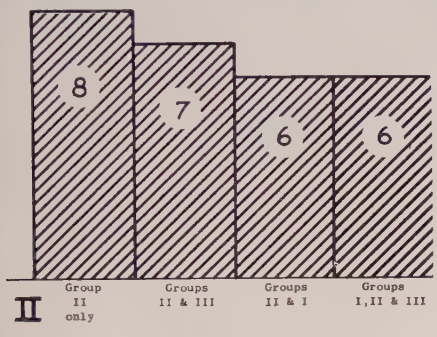


CHART I. Number of sera reacting to mycobacterial antigens according to group (Runyon system).

CHART II. Number of sera showing reactions against antigens of Group II alone, and against antigens of Group II and one or more other groups.

series of ambulant allergy patients, and from a series of patients in a tuberculosis hospital who first were found to have human tubercle organisms in the sputum and later produced various strains of anonymous mycobacteria. Table I presents the results of diffusion studies against the same antigens as used in the cases with sarcoidosis. From 49 allergy patients 5 sera produced reactions with the antigens of mycobacteria but only one of these produced more than a single band. From 111 medical students 28 sera formed bands against the antigens, but again only 4 sera resulted in more than one band, while 15 of the 28 positive sera produced reactions against O.T. as compared with 2 of the 32 sarcoidosis patients.

TABLE I. Diffusion Reactions in Sera from Ambulant, Non-hospitalized Individuals.

	No.	I	II	III	O.T.
Allergic patients	49	1	3	2*	10
Medical students	111	12	12	6†	37

* 1 patient reacted with a single band each to Group I and III.

† 2 sera reacted to more than one group antigen.

Roman numerals in this table refer to anonymous mycobacterial groups. Arabic numerals refer to No. of sera presenting reactions. More than a single band against one of the group antigens was encountered once among the allergy patients and 4 times among medical students.

The sera of 23 patients in a tuberculosis hospital were subjected to the same diffusion studies. These patients had all had sputum cultures positive for human strains of *M. tuberculosis,* but had subsequently had positive cultures for certain of the anonymous mycobacteria. All of these formed zones of precipitation (one, as many as 5) against antigens of H37Rv. Against anonymous antigens, 15 sera produced bands, inconsistent single zones only in the case of the individuals producing Group I or Group III organisms, but more consistent reactions in the case of Group II.

The distribution of reactions among patients with sarcoidosis is different from that of the 3 control groups, mostly nearly resembling the reactions of individuals who produce Group II organisms. Three possibilities can be considered in relation to the presence of mycobacterial antibodies in sera of patients with sarcoidosis: 1) The sarcoidosis patient, as a result of severe stimulus to the reticuloendothelial system, releases a great many antibodies not directly related to the agent. The results of immunization experiments in sarcoidosis patients and the absence of fungal antibodies in the group under study make this explanation unlikely. 2) The antibodies against anonymous mycobacteria may represent cross-reacting antibodies against an agent not represented in the diffusions. 3) The demonstrated antibodies may be specific. In this case their demonstration is of some diagnostic value, if this work can be confirmed, and may have etiological implications.

Conclusions. 1. A serological reaction has been obtained between sera of patients with sarcoidosis and antigens from anonymous

mycobacteria, using agar diffusion technic. 2. The reaction has occurred in sera of 28 of 32 patients, 3 of which changed from negative to positive as lesions improved. 3. The data permit no conclusion as to specificity of this reaction, but suggest its value as an additional diagnostic method in sarcoidosis.

The author expresses gratitude to Dr. M. M. Cummings, Oklahoma City; Dr. Helen Dickie, Madison, Wis.; Dr. Edwin Kendig, Richmond, Va.; and Dr. Howard Buechner, New Orleans, for assistance in obtaining sera from their several areas.

The author wishes to express his great obligation to Miss Maro Speight, whose untiring and enthusiastic efforts have made this study possible, and to Dr. Lloyd Hirshberger, San Angelo, Texas, for providing sera and information on the sanatorium patients.

1. Long, E. R., *Am. Rev. Tuberc.,* 1957, v75, 852.
2. Refvem, O., *Acta, Med. Scand.,* (Supp. 294), 1954, v149, 11.
3. Siltzbach, L. E., *Am. J. Med.,* 1957, v22, 841.
4. Parlett, R. C., Youmans, G. P., *Am. Rev. Tuberc.,* 1958, v77, 450.
5. Heiner, D. C., *Pediatrics,* 1958, v22, 616.
6. Crowle, A. J., *J. Lab. & Clin. Med.,* 1958, v52, 784.
7. Dewlett, H. J., Lefkowitz, L., Fulayfil, R., Christianson, L., Chapman, J. S., to be published.
8. McCuiston, C. F., Hudgins, P. C., *Am. Rev. Resp. Dis.,* 1960, v82, 59.

Received March 28, 1961. P.S.E.B.M., 1961, v107.

Chemical Interaction of S^{35}-6-Mercaptopurine and Ribonucleic Acids.* (26615)

HANS J. HANSEN AND SAMUEL B. NADLER
Departments of Biochemical Research, Touro Infirmary and Dept. of Medicine, Tulane School of Medicine, New Orleans, La.

The question as to whether S^{35}-labelled 6-mercaptopurine. (S^{35}-6 MP) is incorporated into nucleic acids has not been settled. Elion *et al.,* injected mice with S^{35}-6 MP and within 4 hours found the label associated with RNA and DNA isolated from visceral organs(1). Brockman has recently questioned this observation as indicating incorporation of 6-mercaptopurine and states that one would not expect 6-MP to be incorporated into nucleic acid because it is an analog of hypoxanthine and not an analog of adenine or guanine(2). 6-MP, however, is converted to thioxanthine and thiouric acid by mouse tissue and by bacteria(2). It is possible that small amounts of a given dose of 6-MP administered to rats might be converted to thioguanine. Lepage has reported that thioguanine is incorporated into nucleic acid(3). In view of these considerations it was thought worthwhile to examine the nature of incorporated S^{35}-6 MP in rat liver RNA.

In vivo incorporation of S^{35}-6 MP into rat liver RNA was accomplished as follows: Three groups (3 rats per group) of adult white male rats were injected intraperitoneally with 2 mg S^{35}-6 MP. After 4 hours the rats were killed; the livers were removed and frozen in dry ice. They were homogenized in a Waring blender and RNA was isolated by the Kirby phenol extraction method(4). The liver RNA from each of the rats in the first group was assayed for radioactivity in an open window gas flow well counter. Fifty mg liver RNA from each of the rats in the second group were dissolved in 5 ml H_2O and treated with an equal volume of 10% trichloracetic acid at 5°C. The precipitate was collected by centrifugation, washed with 95% EtOH and the radioactivity per mg RNA was measured. Fifty mg liver RNA from each of the rats in the third group were dissolved in 5 ml of water containing 100 μg unlabelled 6-MP and aerated with H_2S gas for one hour at room temperature. The

* This study was supported in part by the John A. Hartford Foundation and in part by the Greater New Orleans Cancer Assn. and Grant CY5689 of N.I.H., Bethesda, Maryland.

RNA was precipitated with 2 volumes 95% EtOH, dialysed against saline for 24 hours and radioactivity per mg RNA was measured. Average amount of mμg S^{35} per mg liver RNA was 4.3, 4.1 and 2.1 respectively for RNA from the livers of rats in Groups 1, 2 and 3. These data indicate that at least half of the S^{35}associated with the RNA fraction could be removed by H_2S treatment at room temperature but was not split off by precipitation with trichloracetic acid at 5°C.

An attempt was made to increase the amount of S^{35} associated with purified extracted rat liver RNA. Each of 3 groups of 3 rats was given 5 mg S^{35}-6 MP intraperitoneally every day for 3 days. Rat liver RNA was isolated as described for Rat Group 3 above. The livers of these 3 groups showed an average of 3.0, 2.3, and 1.1 mμg S^{35} per mg RNA. There was no significant increase in associated S^{35}. These data confirm the experimental results of Elion, Bieber and Hitchings in that S^{35} is associated with purified isolated liver RNA after injection of S^{35}-6 MP into rats. However, the findings with H_2S suggest that part of the "incorporated" S^{35} may be bound as a mercaptide.

Two types of experiments demonstrated that 6-MP combines with RNA to form a stable complex. It was found that S^{35}-6 MP combines with RNA of homogenized rat liver and that it combines with pure yeast RNA to form a stable complex.

1. Combination of S^{35}-6 MP with rat liver RNA. Ten grams of rat liver from each of 3 rats were separately homogenized in a Waring blender for one minute with 4 volumes of water containing 5 mg S^{35}-6 MP. RNA was isolated by the Kirby method and aerated with H_2S for one hour at room temperature. The RNA was precipitated with 2 volumes of 95% EtOh, dialysed against saline for 24 hours and radioactivity per mg RNA was measured. 2.1, 1.1 and 0.2 mμg S^{35} were associated with the liver RNA fractions. Two or 3 times as much S^{35} per mg RNA was present prior to H_2S treatment. A small but measurable fraction appears to resist separation by H_2S.

2. Combining of yeast RNA with S^{35}-6 MP.† Commercial yeast RNA was obtained from Nutritional Biochemical Corp. Fifty aliquots were dissolved in 5 ml of 2% KAc solution. One tube served as control, while to the others were added copper and iron salt solutions in the amounts of 1 μm/50 mg RNA. Two volumes of alcohol were added and the resulting precipitates of RNA were collected by centrifugation. The precipitates were dissolved in 5 ml of 2% KAc and alcohol precipitation was repeated. The precipitates were re-dissolved in 5 ml of 2% KAc solution containing 100 μg of S^{35}-6 MP per 5 ml salt solution. After 15 minutes RNA was precipitated with alcohol, the precipitate collected by centrifugation and re-dissolved in 5 ml of 2% KAc. This step was repeated 4 times to remove free S^{35}-6 MP. The RNA was assayed for S^{35} per mg RNA. The results given in Table I represent averages of 4 such experiments.

TABLE I.* Effects of Metals on Combining of S^{35}-6 MP by Yeast RNA.

Metal added	mμg S^{35}/mg RNA
None	13
Cu^{+}	133
Cu^{++}	95
Fe^{++}	35
Fe^{+++}	13

* Avg of 4 experiments.

Discussion. When S^{35}-6 MP is injected into rats, small amounts of radioactivity are associated with liver RNA isolated by the Kirby procedure involving phenol extraction, alcohol precipitation and dialysis. Since at least half of the radioactivity can be removed by treatment with H_2S at room temperature, it would appear that RNA may be combined with 6-MP by a metal bridge bond as shown below:

RNA - Metal - S-6 MP

The same reaction appears to take place immediately when rat liver is homogenized in presence of S^{35}-6 MP—a speed of reaction which would seem to preclude "incorporation". Commercial yeast RNA also com-

† S^{35}–6 MP was synthesized in this laboratory by methods and materials kindly supplied by Dr. George Hitchings, Burroughs Wellcome Lab., Tuckahoe, N. Y.

bines with small quantities of S^{35}-6 MP and addition of metals (Table I) enhances the combination. Various metals have been reported to be combined with nucleic acids(5, 6). The interaction of 6-MP with metals in nucleic acid may interfere with cellular metabolism.

Summary. These data indicate that 6-mercaptopurine can combine with ribonucleic acid both *in vitro* and *in vivo*. Binding is enhanced by added metals and the combination is stable to ribonucleic acid isolation procedures.

1. Elion, G. B., Bieber, S., Hitchings, G. H., *Ann. N. Y. Acad. Sci.,* 1954, v60, 297.
2. Brockman, R. W., *Cancer Res.,* 1960, v20, 643.
3. Le Page, G. A., *ibid.,* 1960, v20, 403.
4. Kirby, K. S., *Biochem. J.,* 1956, v64, 405.
5. ———, *ibid.,* 1957, v66, 495.
6. Wacher, W. E. C., Vallee, B. L., *J. Biol. Chem.,* 1959, v234, 3257.

Received April 3, 1961. P.S.E.B.M., 1961, v107.

Studies on Murine Hepatitis Virus (MHV3) *in vitro.** (26616)

TAPANI VAINIO (Introduced by David Kritchevsky)
The Wistar Institute, Philadelphia, Pa.

The results of several investigations from various approaches(1,2) have focused attention upon murine hepatitis virus and have indicated the possible use of studies of these viruses *in vitro*.

Until recently, knowledge of the murine hepatitis viruses has been based mainly on experiments *in vivo,* since in spite of the observations that these viruses multiply in tissue culture(3-5), no cytopathic effect has been demonstrated. However, Bang(6) has recently shown that MHV2 (Nelson virus) destroys macrophages spreading out from liver explants obtained from a strain of Princeton mice susceptible to hepatitis virus. Parallel observations were made by Bang (7) in macrophage cultures from Princeton mice which were lysed by the virus; macrophages from resistant C3H mice showed no cytopathic effect. This report concerns the behavior of another strain of murine hepatitis (MHV3) in tissue culture, since this strain has been used in experiments designed to show the protective action of antihistamines against liver injury(8,9).

Methods. 1. *Virus.* The MHV3 virus, obtained from Dr. Gledhill, was injected intraperitoneally into adult C57Bl/6 mice, and the infected livers were harvested from moribund mice. The livers were pale and under histological examination appeared to be completely necrotic. For experiments *in vitro* viral pool suspensions were prepared from 10% homogenates of these livers. The homogenate was centrifuged at 1500 rpm for 15 minutes and the supernatant diluted 10-fold with culture medium.

2. *Tissue culture.* Liver explants from 16 to 20-day old fetuses of C57Bl/6 mice were cultivated on reconstituted rat-tail collagen as described by Hillis *et al.*(10). Collagen was prepared and stored according to Ehrmann *et al.*(11). Fetal livers pooled from the same litter were minced with scissors, and 0.5 mm^3 pieces were placed on coverslips on collagen, which were placed in roller tubes. Rotation speed was adjusted to 8 rph. Culture medium consisted of 30% active horse serum, 5% chick embryo extract, and 65% Eagle's basal medium in Earle's balanced salt solution. Tubes were gassed with 5% CO_2 before being stoppered.

In addition, dispersed spleen cell cultures were prepared according to the method of Manaker *et al.*(12).

3. *Passage of virus in tissue culture.* Virus was passed in tissue culture either by using 10-fold diluted infected culture medium or by transferring homogenates from infected explants. This was accomplished by breaking

* This work was supported by grant from Nat. Inst. Health, P.H.S.

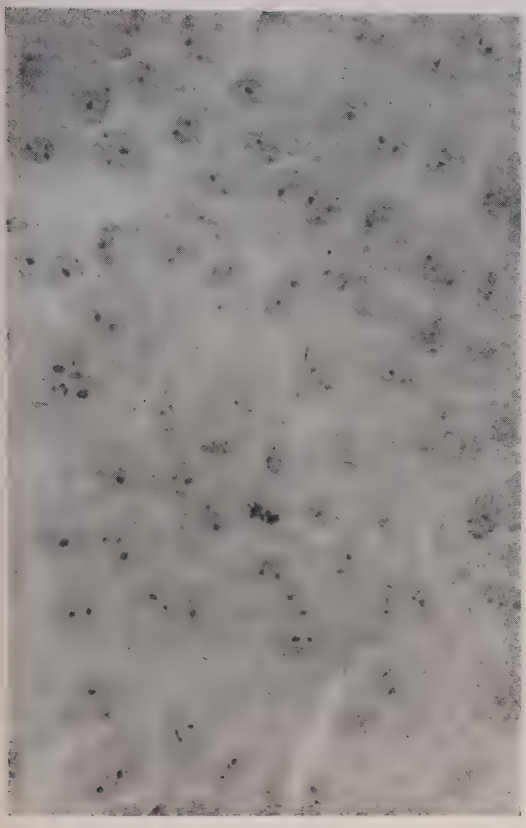


FIG. 1. Epithelial outgrowth from fetal liver explant cultivated on reconstituted collagen. × 250.

the explants, each of which was suspended in 1 ml of saline, in a sonic oscillator. This material was diluted by a factor of 10 and transferred into fresh cultures. Infectious material was carried serially from infected cultures to fresh culture by diluting it directly in culture medium of a new explant.

4. *Inoculation of mice.* Virus from explants was assayed *in vivo* using the infected tissue. Presence of virus in tissue culture passages was determined by inoculation of mice with either media or with washed explants prepared as described earlier. One-tenth ml of the preparation was injected intraperitoneally into adult C57Bl/6 mice.

5. *Preparation of antisera.* Antisera against MHV3 and MHV2 (Nelson virus) were prepared in C57Bl/6 and Princeton (PRI) adult mice by challenging those surviving from the primary infection with an intraperitoneal injection of virus from infected explants. Blood was collected by cardiac puncture one week after the second injection, pooled, and inactivated for one-half hour at 56°C. Serum was separated after overnight refrigeration and was stored at –20°C.

6. *Histological examination.* For histological studies, explants were fixed in Zenker's fluid and stained routinely with hematoxylin and eosin without being removed from the coverslip. This procedure permits detailed study of the outgrowth but not of the explant itself.

Results. Liver explant on collagen. It is known that fetal liver does not survive long when cultivated on a plain glass surface. Collagen enhances outgrowth from liver explants(7,10) and this outgrowth includes either palisades, or sheets of epithelial cells which have the appearance of liver parenchyma (Fig. 1). Growth sequence of cells from the explant is as follows. After one or 2 days in cultivation, fibroblasts start to spread out. Coincidently a number of free cells scatter in the vicinity of the explant, some of which reach and attach themselves to the walls of the culture tube. In subsequent days epithelial cells start to grow out mainly in sheets, sometimes as palisades. Epithelial outgrowth often reaches and passes the edge of the fibroblastic outgrowth. During this time, free cells around the explant increase in number; are phagocytic and motile, and resemble macrophages.

Effect of MHV3 on fetal liver cultures. When a homogenate prepared from an MHV3-infected liver was added to cultures, a cytopathic effect appeared in 3-5 days which was easy to distinguish from the gross appearance of the explant (Fig. 2A & B). Fibroblastic outgrowth appeared to be little affected but epithelial cells tended to form clumps, become detached, and then lysed. Closer examination revealed that most of the epithelial outgrowth was destroyed; the cells showed various stages of pyknosis and in areas of worst damage the liver cells disappeared, leaving the fibroblasts (Fig. 3). When tested with neutral red (1:40,000 dilution) outgrowth from infected cultures picked up the stain far less than that of the control cultures (Fig. 4A & B). As shown

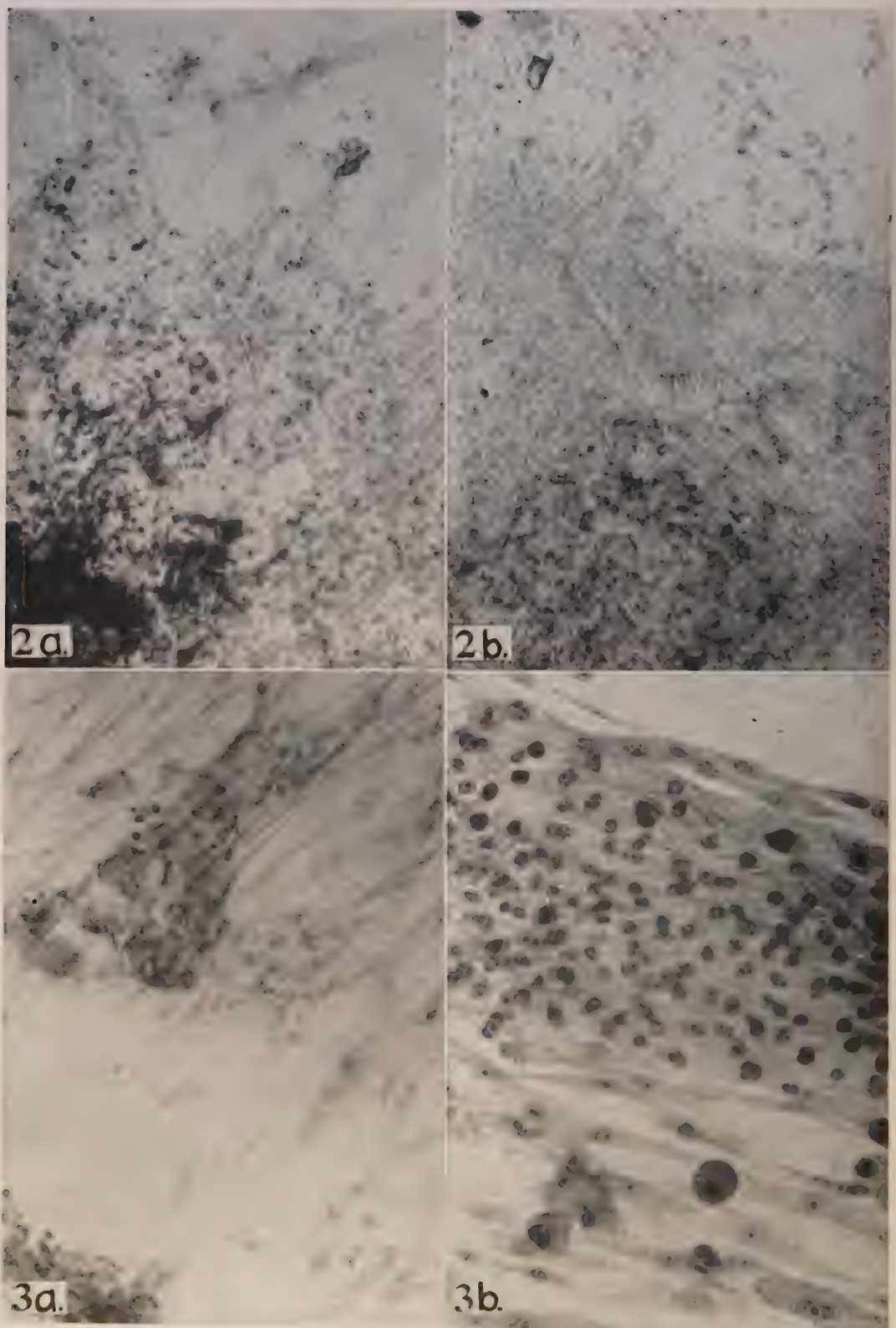


FIG. 2. Gross appearance of liver outgrowth from (a) infected and (b) control cultures. × 35.
FIG. 3. Epithelial outgrowth after 4 days infection with (a) MHV and (b) that of control culture stained with hematoxylin and eosin. × 125.

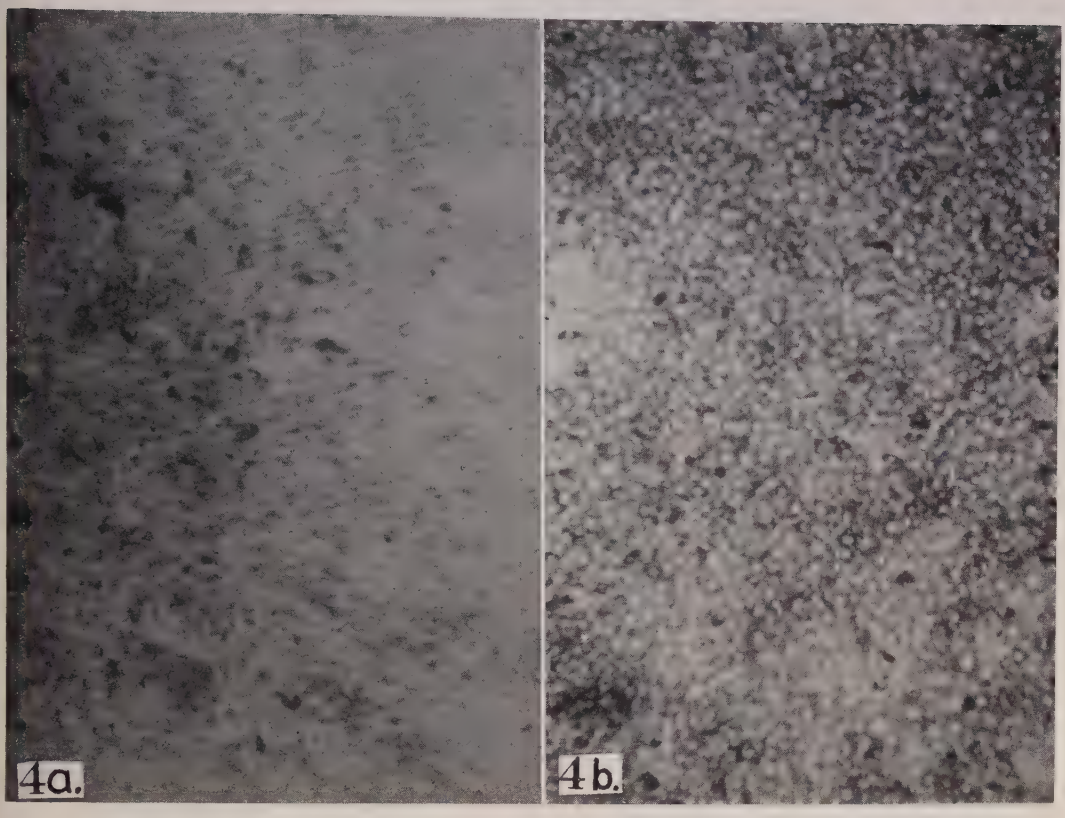


FIG. 4. MHV-infected liver explant culture (5 days from inoculum of virus) (a) stained with neutral red, and (b) control culture. × 125.

by Dick *et al.*(13), pleomorphic basophilic cytoplasmic inclusions were observed by means of the histological technic described (Fig. 5).

As infection advanced in explants, phagocytic cells became affected. To determine whether these cells were attacked directly by virus, macrophage cultures prepared from spleen were exposed to infectious tissue culture fluid from explant cultures. The macrophages were destroyed in 2-3 days.

Transmitting MHV3 virus and assay in vivo. MHV3 virus was carried in fetal liver explant cultures for 22 passages by serial transfer of tissue culture fluids and/or explant homogenate from cultures showing advanced CPE to newly prepared liver explants. Infectious material was usually harvested 3 days after inoculation, but seemed to yield virus at least 5 days thereafter when tested on fresh cultures. From time to time infected culture material was injected intraperitoneally in adult mice and lethal amounts of virus were shown both in the culture medium and in the explant itself (Table I). Some explant cultures were apparently able to support the multiplication of virus, but did not show CPE. It appeared that explants from certain litters were resistant to cytopathic action of the virus. No explanation for this variation is offered because the amount of virus transferred was not measured.

Strain specificity of MHV3. A few strains of mice other than C57Bl/6 were tested and lesions were observed after liver explants from C3H, BRVR, and BSVS fetuses were infected with MHV3. No differences in time of appearance and nature of the lesion were seen as compared with cultures from C57Bl/6 mice. Macrophages derived from spleens of one-month old C3H and C57Bl/6 mice were found to be highly susceptible to MHV3 virus.

TABLE I. Passage of MHV3 in Fetal Liver Explant Cultures and Assay of Infected Culture Material *In Vivo*.

No. passage	Culture material treated with	CPE No. of positive/No. of infected cultures	*In vivo* assay Material	No. mice dead/No. mice inj.
3	None	7/12	Explants	5/5
			Media	9/10
3	"	0/7	Explants	4/5
			Media	5/5
14	"	16/16	Explants	6/10
			Media	9/10
21	Normal mouse serum 1/100	7/7	Explants	3/5
			Media	2/5
21	Mouse anti-MHV3 1/100	0/7	Explants	0/5
			Media	0/5

Inactivation of cytopathic activity of virus. MHV3 virus obtained from liver explants is rapidly inactivated at 37°C. Thus only a few of the tested cultures were destroyed when the infectious material was previously inactivated for 60 minutes at 37°C; and no cytopathic effect was observed after inoculation of infectious material previously maintained at 37°C for 3 hours.

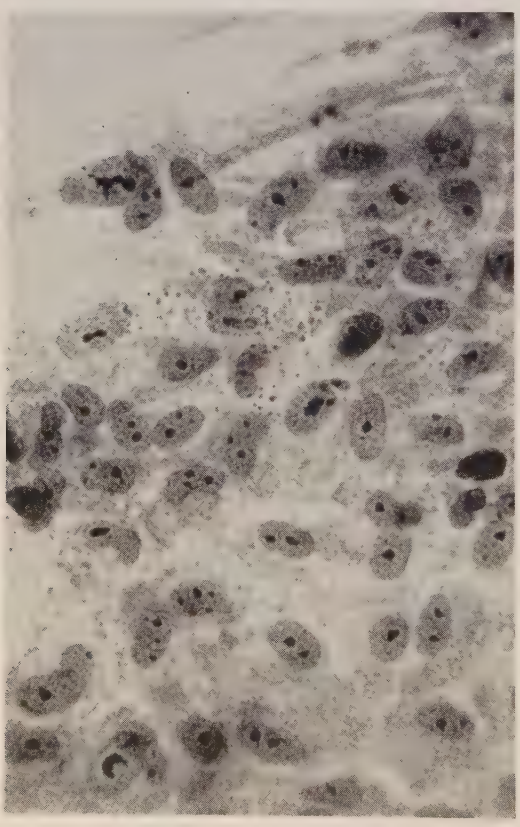


FIG. 5. Basophilic inclusions in epithelial cells after 2 days MHV infection. × 160.

Recovery of virus from infected cultures. Yields of MHV3 virus from infected cultures were low. Only on 2 occasions has a 10^{-2} dilution of a liver explant homogenate given rise to lesions in fresh cultures.

Certain factors did not alter in any way the cell damage caused by MHV3 virus on liver explants. They were: age of fetus from which the explant was prepared; age of the culture itself, if infected before the 14th day of cultivation· use of calf serum instead of horse serum and omission of chick embryo extract from the medium.

Effect of storage on viral activity. Virus in infected explants remained infective after storage at –20°C for at least 2 months. In infected culture medium, viral activity was often lost under these conditions, although occasionally activity was observed after storage of culture medium at –70°C for one month·

Effect of antisera. Contrary to earlier observations(13,14), sera of animals which had been twice injected with infectious material inhibited CPE in high titer. A dilution of 10^{-4} of anti MHV3 mouse serum prevented cytopathic effect of MHV3 virus in liver explants. Control sera from individuals of the same strain occasionally inhibited CPE only when used undiluted. *In vivo* assays of cultures in which viral cytopathogenicity was inhibited (Table I) revealed no virus.

As described earlier(9), pooled serum from mice twice-infected with MHV2 inhibited cytopathic effect caused by MHV3 virus. Under the experimental conditions used, the inhibitory titer of pooled serum from immunized PRI mice was 10^{-2}.

Discussion. Working with Nelson virus (MHV2), Bang *et al.*(6,7) showed that macrophages were destroyed *in vitro*. Although no lesions were found in liver cells, infected cultures produced less acid than the controls. Among other hepatitis viruses, only that of the duck has been shown to damage liver parenchymal cells *in vitro*(15). Human hepatitis virus is unable to affect human fetal liver explant cultivated on reconstituted collagen(10). The cytopathic effect demonstrated in liver parenchymal cells and in the area of epithelial outgrowth of murine fetal liver explant is the only *in vitro* hepatocytocidal effect due to hepatitis viruses in mammals described so far. The only noticeable difference between the technics used by Bang and those used in this work lies in the amount of collagen employed. In the experiment above, the collagenized coverslip instead of collagen slant in roller tube was used. Bang(16) has suggested that the amount of collagen might be of importance in demonstration of the hepatocytocidal effect of hepatitis viruses.

Even though semiquantitative technics were used throughout these experiments, it became evident during the study that MHV3 can multiply in some explant cultures without causing cellular damage. Since this resistance seemed to be a property of the cells from all livers in the same litter it is suggested that liver cell necrosis is indirectly related to viral multiplication, being brought about by the operation of a secondary mechanism which in CPE-resistant livers is not set into motion. It is believed that the mechanism of liver cell necrosis is inhibited by anti-histamine drugs. This possibility is dealt with elsewhere(17).

A low level of activity of antisera against murine hepatitis viruses has been demonstrated so far. The high neutralizing titers demonstrated in the sera of mice immunized against MHV3 were, therefore, unexpected. It remains to be determined whether this high titer of neutralization is due solely to the antibodies or whether some other factors are involved.

Summary. It has been shown that murine hepatitis virus (MHV3) can be propagated serially in liver explant cultures. Cultures were prepared from fetal C57Bl/6 livers and cultivated on rat-tail reconstituted collagen. MHV3 virus caused cytopathic changes in such explants that are mainly in the epithelial outgrowth and macrophages. Virus has been carried for 22 passages and has been recovered by assaying the culture material in mice. Using this system, the sera from mice infected with the virus have been tested and high titers of CPE inhibitory activity were demonstrated.

1. Huebner, R. J., *Cancer Research,* 1960, v20, 786.

2. Nelson, J. B., *Bull. N. Y. Acad. Med.,* 1957, v33, 2nd ser., 811.

3. Starr, T., Pollard, M., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 97.

4. Gompels, A. E. H., Niven, J., *J. Path. Bact.,* 1952, v66, 567.

5. Miuazaki, Y., *et al., Japan. J. Exp. Path.,* 1957, v27, 381.

6. Bang, F. B., Warwick, A., *Virology,* 1959, v9, 716.

7. Bang, F. B., *Proc. Nat. Acad. Sci. Wash.,* 1960, v46, 1065.

8. Gallagher, C. H., *et al., J. Path. Bact.,* 1958, v72, 1.

9. Judah, J., Bjotvedt, G., Vainio, T., *Nature,* 1960, v187, 507.

10. Hillis, W. D., Bang, F. B., *Exp. Cell Res.,* 1959, v17, 557.

11. Ehrmann, R. L., Gey, G. O., *J. Nat. Cancer Inst.,* 1956, v16, 1375.

12. Manaker, R., Strother, P. C., Miller, A., Piczak, V., *ibid.,* 1960, v25, 1411.

13. Dick, G. W., Niven, J., Gledhill, A. W., *Brit. J. Exp. Path.,* 1956, v37, 90.

14. Gledhill, A. W., Dick, G. W., Niven, J., *J. Path. Bact.,* 1955, v69, 299.

15. Bang, F. B., *Hepatitis Frontiers,* 1959, Boston.

16. ———, personal communication.

17. Vainio, T., Judah, J., Bjotvedt, G., to be published.

Received April 3, 1961. P.S.E.B.M., 1961, v107.

Interactions of *Vibrio cholerae, Shigella flexneri,* Enterococci, and Lactobacilli in Continuously Fed Cultures. (26617)

JOHN P. RANSOM,* R. A. FINKELSTEIN, ROBERT E. CEDER AND SAMUEL B. FORMAL
Division of Communicable Disease and Immunology, Walter Reed Army Institute of Research, Washington, D.C.

The factor or factors precipitating signs and symptoms of clinical cholera in man are not well-defined. Attributes of the organism itself have been repeatedly invoked in the 76 years since Koch's isolation of the vibrio. Lankford(1) has recently reviewed the significant findings in this field. Similarly, Pollitzer(2,3) has reviewed the literature with respect to characteristics of the host which may be of significance in susceptibility of man to infection with *Vibrio cholerae.*

The possibility has been considered that the normal flora of the intestinal tract might be significant in susceptibility to infection with enteric pathogens. Freter(4,5) has demonstrated that infections with *Shigella flexneri* and *V. cholerae* could be established in the intestines of normally resistant mice and guinea pigs when the intestinal microflora were reduced or eliminated by previous treatment with antibiotics, and that such infections could be eliminated by the simultaneous administration of viable cells of *Escherichia coli*(5,6). However, cholera is an infection of the small intestine and Lankford(1) has pointed out that the absence of *E. coli* from this region in man and laboratory mammals would cast doubt upon its presumed ability to prevent infection by direct antagonism.

Unlike *E. coli,* lactobacilli are common inhabitants of the upper as well as the lower intestinal tract of laboratory animals(9,10, 11). The same is also true of Group D streptococci or enterococci, though perhaps not universally present or present in lesser numbers(9,10,11). In addition, lactobacilli have been reported to influence the course of infection(7) or to be capable of producing antibiotics(8). We therefore selected these 2 types of organisms for a study of interactions *in vitro.*

The technic of continuous culture chosen to carry out this study was used by Freter (6,11) to evaluate interactions between strains of coliform bacteria and enteric pathogens. He found inhibition of shigellae and cholera vibrios by this means when other methods failed. These findings emphasize the importance of studying bacterial interactions in an environment dynamically similar to the native ecological niche. This is particularly true with regard to enteric pathogens which multiply and cause their effects in the lumen of the gut, where conditions are constantly changing; new nutritional substances continually are coming in, while spent materials and parts of the bacterial population are departing. In this paper certain interactions of indigenous biota of the intestine and 2 enteric pathogens, *Vibrio cholerae* and *Shigella flexneri* 2a are examined.

Methods and materials. The apparatus designed for continuous culture(17) embodies a number of principles set forth by others(12, 13, 14, 15, 16). The general subject has been reviewed by Novick(16). A diagram of the apparatus used in this work is given in Fig. 1.

The experiments were conducted by setting up pairs of growth tubes, each receiving an identical flow of fresh medium, and draining through rubber tubing into a common waste receptacle. Under aerobic operations, the glass tube (K, Fig. 1) was plugged with cotton and used as a sampling port. When anaerobic operation was desired, this tube was closed with a vaccine vial stopper. A flow of nitrogen was then introduced through a heavy gauge (18 or 19 ga.) hypodermic needle inserted into the vaccine vial stopper. A steady flow of gas (5% CO_2, 95% N_2) had no deleterious effect on the operation of the system.

Flow rates were adjusted to 74-76 ml/hr.

* Present address: New England Inst. for Medical Research, Ridgefield, Conn.

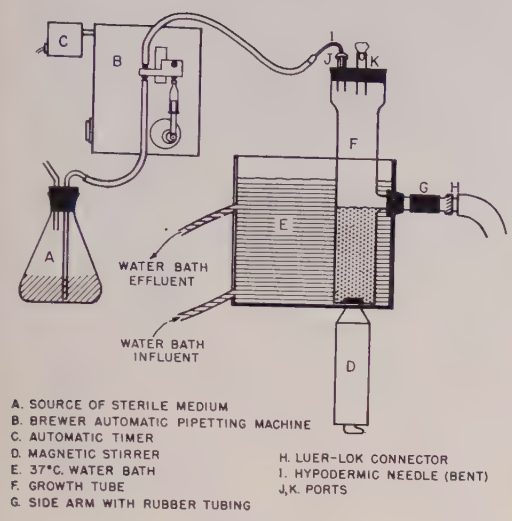


FIG. 1. Diagrammatic sketch of continuous culture apparatus.

The effective capacity of the growth tubes used was 79-81 ml. Thus, the turnover of medium in the growth tubes was approximately 100% per hour. Under these conditions, samples were taken by puncturing, with aseptic technic, the rubber tubing connected to the side-arm, using a sterile hypodermic needle and syringe.

Cultures: Vibrio cholerae (Inaba) strain 20-A-67 was obtained from the WRAIR collection. *Shigella flexneri* 2a strain 2457 was isolated in 1954 by Lt. Colonel Oscar Felsenfeld from a dysentery outbreak in Japan. They were maintained on meat extract agar kept at 5°C. Transfers of the stock cultures were made at weekly intervals. Inocula for continuous culture experiments consisted of 1 ml of a dilution of agar grown suspension of organisms containing 10^2 to 10^3 viable cells.

The enterococcus culture employed was a Group D streptococcus isolated from a human source. The culture was provided by Dr. James Rust, Dept. of Bacteriology, WRAIR.

The culture designated as "lactobacillus" was isolated from a normal guinea pig small intestine. This organism grows readily on a selective medium(18), is peroxidase-negative and cytochrome-negative as tested by the method of Deibel and Evans(19). The organism is a Gram-positive, non-spore-forming, non-motile rod. It grows poorly under strictly aerobic conditions. The criteria mentioned above are considered sufficient to identify this organism as a lactobacillus type.

Growth medium: The medium used for growth in continuous culture was essentially Bacto-Thioglycollate Medium. The agar was omitted from this medium and glucose was added to a concentration of 0.1%. In addition, the medium was buffered with phosphate buffer to a pH of 6.9-7.2 after autoclaving. This step was found necessary to maintain the pH at suitable levels.

Recovery media: For plate counts of *V. cholerae* and *Sh. flexneri* 2a, a meat extract agar of the following composition was used:

Beef extract	3 g	NaCl	5 g
Bacto-peptone	10 g	2N NaOH	3 ml
Bacto-agar	20 g	Distilled water	1000 ml

For the enterococcus, Brain Heart Infusion Agar (BHIA) (Difco) was used.

For the lactobacillus, the Lactobacillus selection medium of Baltimore Biological Laboratories, Inc. was employed.

All plate counts were performed by the method of Miles *et al.*(20). The plate counts for *V. cholerae* were incubated 18-24 hours aerobically at 37°C. Under these conditions, the lactobacilli did not grow at all within this time period, and the enterococci, although forming a slight background of growth did not interfere with enumeration of the cholera colonies. Preliminary experiments indicated that *V. cholerae* or *S. flexneri* counts from mixed cultures were equivalent to those obtained in pure culture. The coefficient of variation of the method ranged from 3 to 30%.

More difficulty was encountered in enumerating enterococcus colonies on BHIA. On this medium the vibrio or shigella colonies grew as well as the enterococci; however the latter were readily distinguished on the basis of colony morphology. Preliminary studies using a selective medium for the enterococci (SF medium, Difco) indicated that counts of the enterococcus on BHIA in presence of vibrio or shigellae were equivalent to those obtained on the selective medium. The latter

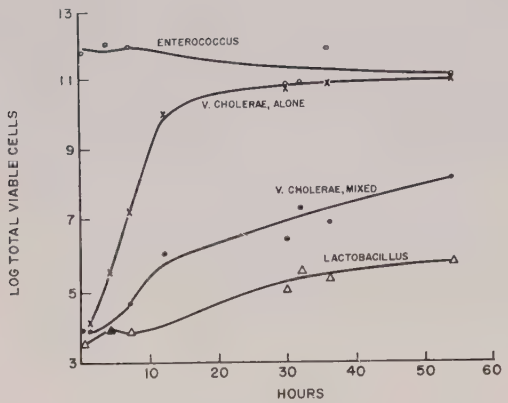


FIG. 2. Effect of enterococcus and lactobacillus growth of *Vibrio cholerae* in continuous culture.

was not employed because of the long incubation period (48-72 hours) necessary to obtain a count.

Plate counts of lactobacilli made on LBS medium were incubated 48 hours at 37°C in candle jars. Under these conditions, the other organisms produced no visible growth.

Static Stirred Cultures: For certain experiments, pure and mixed cultures were grown in 25 × 200 mm test tubes. Magnetic stirring bars were always used to maintain homogeneity of the cultures. Conveniently, these tubes could be placed in the water bath of the continuous culture apparatus over the magnetic stirring motor.

Chick Embryo Studies: Experiments with *V.* cholerae and the enterococcus culture were carried out in the chick embryo employing procedures already described(21).

Results: In the first experiment, the enterococcus and the lactobacillus strains were grown together in one tube of the continuous culture apparatus until each reached a steady state. At this time, equal numbers (10^2-10^3) of *V. cholerae* were added to the mixed culture and also to an uninoculated tube. The numbers of organisms in each tube were determined over a 54 hour period. Results are summarized in Fig. 2. Throughout the 54 hour period of observation, the numbers of viable cells of enterococci remained high and almost constant (*ca.* 10^{12} cells total). The lactobacilli, however, remained at low levels (10^4-10^5), but increased slightly between 36 and 54 hours.

The *V. cholerae* cells in pure culture went into a phase of exponential growth after a short lag period of approximately one hour, and reached a steady state after 12 hours at 10^{10}-10^{11} cells. One must bear in mind in interpreting Fig. 2, that the cells were not only increasing in numbers or remaining constant, but were keeping up with the constant influx of fresh medium and loss of some cells, amounting to nearly a complete turnover in volume of the growth tube each hour. Table I shows the calculated generation times required for each organism to achieve the performance shown in Fig. 2.

In the mixed culture, the *V. cholerae* growth exhibited a longer delay, followed by a gradual increase to a level of 10^6-10^7 cells, a level some 10,000-fold lower than that achieved by sister cells in pure culture.

Although the culture containing the mixed growth of enterococci and lactobacilli maintained a reasonably constant pH of 6.5-6.6 during the preliminary period of incubation, it showed some fluctuation after addition of the vibrios, and was not always identical with the pure vibrio culture. Therefore, an experiment was conducted in static stirred cultures to determine the effect of various pH levels on growth of the vibrio. Aliquots of medium were adjusted to pH 7.00, pH 6.32, and pH 6.12 with phosphate buffer and seeded with

TABLE I. Generation Times of *Vibrio cholerae* in Continuous Culture.

Time (hr)*	Generation times (min.)† Pure	Mixed with enterococcus
0 – 1	57.7	52.6
1 – 3½	16.0	43.4
3½–7	14.3	29.5
7 –12	17.3	24.2
12 –30	44.7	42.0
30 –36½	43.6	42.0
36½–54	43.6	35.6

* After inoculation of *V. cholerae*.

† Time required for population to double, including correction for flow rate, *i.e.*

$$\text{Doubling time} = \ln 2 \Big/ \frac{\ln N_2 - \ln N_1}{t_2 - t} + \frac{w}{v},$$

where N_2 = population at time t_2
N_1 = population at time t
w = flow rate
v = vol of growth tube

TABLE II. Effect of Variation of pH on Growth of *Vibrio cholerae* in Static Culture.

Time after inoc. (hr)	Viable cells/ml Starting pH of medium 7.00	6.32	6.12
0	1.00×10^3	1.00×10^3	1.00×10^3
2	4.45×10^2	4.45×10^2	1.78×10^2
6	5.30×10^6	1.47×10^6	3.26×10^5
13	6.82×10^8	6.45×10^8	6.25×10^8
28	4.90×10^8	5.58×10^8	7.35×10^8

identical inocula. The results are shown in Table II. The slight differences observed were not sufficient to account for the results obtained in continuous culture.

A second experiment was carried out in continuous culture to determine if enterococci alone could inhibit growth of *V. cholerae* to the degree achieved by the combination of lactobacillus and enterococcus. The results were similar to the experiment summarized in Fig. 2. When the experiment was carried out under anaerobic conditions, inhibition of *V. cholerae* was also observed. Under aerobic conditions, maximum cell yield of the pure culture of cholera was 8.72×10^8 while under anaerobic conditions, maximum cell yield was 1.91×10^9. This difference in the pure cultures is not considered significant. However, maximum cell yield of the *V. cholerae* in mixed culture was 2.77×10^6 under aerobic conditions and 8.55×10^4 cells under anaerobic conditions.

Two experiments similar to those described above were conducted on mixed cultures of enterococci and *Shigella flexneri* 2a, one under aerobic and one under anaerobic conditions. In neither case was a significant difference in the control shigella culture and the combined enterococcus-shigella culture demonstrable.

The ability of the enterococcus to protect embryonated eggs against an experimental cholera infection was tested by technics previously described(21). Twelve-day-old chick embryos were inoculated allantoically with approximately 10^6 enterococcus cells and groups were challenged 24 hours later with graded doses of *V. cholerae*. The data are summarized in Table III. Control eggs inoculated only with dilutions of *V. cholerae* succumbed rapidly; the LD_{50} at 48 hours approximated 3.5 organisms. The enterococcus is not pathogenic for chick embryos and did not kill any eggs. However, previous infection with enterococci conferred a degree of resistance against subsequent challenge with *V. cholerae*. Other strains of group D streptococci exhibit this effect to varying degrees. In a later experiment, no protection was obtained when graded doses of cholera vibrios were inoculated immediately following the enterococci. Thus the results are similar to those of Finkelstein and Ransom (21) with *E. coli* in which it was demonstrated that endotoxin was the resistance promoting factor. To our knowledge there have been no reports of endotoxin-like factors in fecal streptococci. Lactobacilli (9 strains) apparently do not multiply in embryonated eggs and do not protect against *V. cholerae* even when heavy doses were administered 24 hours prior to challenge.

Discussion. A true steady state of exponential growth is usually achieved in pure continuous cultures by control of turbidity (14) or by limiting quantity of essential nutrients(12,13). However, this type of control is difficult to attain in the case of mixed populations in continuous culture.

Thus, the exact status of the organisms multiplying in mixed continuous cultures cannot be precisely defined. Nevertheless, the situation is probably more truly representative of their status in their respective ecological niches than is a static situation in which organisms reach a maximum stationary phase as a consequence of a complete ex-

TABLE III. Effect of Prior Inoculation with Enterococci on Mortality of Embryonated Eggs Infected with *V. cholerae*.

V. cholerae inoculum (log)	Mortality* Control	Enterococci†
10^0	1/8	0/8
10^1	8/8	4/8
10^2	8/8	3/8
10^3	8/8	2/8
10^4	8/8	3/8
10^5	7/7	2/5

* No. dead/total. 48 hr after challenge.

† 10^6 enterococci inoculated 24 hr prior to cholera challenge.

haustion of nutrients or accumulation of toxic end-products of the multiplying cells themselves.

As demonstrated above, certain interactions occur between the bacterial species we have studied which are not readily demonstrated by other techniques on agar medium. *V. cholerae* grew readily on a lawn of growing enterococcus cells. The reverse was also true. Similarly, no inhibition was noted between adjacent colonies of one or the other organism. The technic of Fredericq(22) revealed no antagonism. No antibiotics against *V. cholerae* were demonstrable in 24 hour filtrates of enterococcus cultures. At present, there is no explanation of these effects. It seems unlikely that the turnover rate of 100% during at most 4 generations of the growing enterococcus could permit the latter to reduce significantly nutrient materials available to the vibrios. Elimination of this possibility, however, awaits further study. It appears more likely that suppression of cholera vibrios by enterococci in continuous mixed culture is due to the momentary existence of intermediates of the enterococcus metabolism which are inhibitory to the vibrios.

Summary: An apparatus for studying interactions of bacteria in continuous culture has been described. It was shown that, in the dynamic situation of continuously renewed nutrient, growth of *V. cholerae* is partially suppressed in the presence of growing enterococci and lactobacilli, and that the inhibition was due largely or entirely to the enterococcus and took place under both aerobic and anaerobic conditions.

The fundamental cause of the inhibition of cholera vibrios by enterococci is not known, but is not due to the inhibitory effect of variation of hydrogen ion concentration, and probably cannot be attributed to competition for nutrients.

1. Lankford, C. E., *Ann. N. Y. Acad. Sci.,* 1960, v88, art. 5, 1203.

2. Pollitzer, R., *WHO Monograph,* Cholera, 1959.

3. Pollitzer, R., *WHO Spec. Bull.,* Review of Recent Literature on Cholera, 1961.

4. Freter, R., *J. Inf. Dis.,* 1955, v97, 57.

5. ———, *J. Exp. Med.,* 1956, v104, 411.

6. ———, *Bact. Proc.,* 1959, v59, 97.

7. Ushida, D., Yumito, M., Ohno, S., *Keio J. Med.,* 1955, v41, 163.

8. Vincent, J. G., Veomitti, R. C., Riley, R. F., *J. Bact.,* 1959, v78, 477.

9. Crecelius, H. G., Rettger, L. G., *ibid.,* 1943, v46, 1.

10. Gall, L. S., Fenton, P. R., Cowgill, G. R., *J. Nutrition,* 1948, v35, 13-25.

11. Freter, R., Personal Communication, 1960.

12. Monod, J., *Ann. Inst. Pasteur,* 1950, v79, 390.

13. Novick, A., Szilard, L., *Science,* 1950, v112, 715.

14. Northrop, J. H., *J. Gen. Physiol.,* 1954, v38, 105.

15. Formal, S. B., Baron, L. S., Spilman, W., *J. Bact.,* 1956, v72, 168.

16. Novick, A., *Ann. Rev. Microbiol.,* 1955, v9, 97.

17. Rockenmacher, M., Ransom, J. P., 1960, unpublished observations.

18. Rogosa, M., Mitchell, J. A., Wiseman, R. F., *J. Bact.,* 1951, v62, 132.

19. Deibel, R. H., Evans, J. B., *ibid.,* 1960, v79, 356.

20. Miles, A. A., Misra, S. S., Irwin, J. O., *J. Hyg.,* (Cambridge), 1938, v38, 732.

21. Finkelstein, R. A., Ransom, J. P., *J. Exp. Med.,* 1960, v112, 315.

22. Fredericq, P., *Ann. Rev. Microbiol.,* 1957, v11, 7.

Received April 4, 1961. P.S.E.B.M., 1961, v107.

Serum Binding, Distribution and Excretion of Four Penicillin Analogues Following Intravenous Injection in Man.* (26618)

CALVIN M. KUNIN (Introduced by William S. Jordan, Jr.)
Departments of Preventive Medicine and Medicine, University of Virginia School of Medicine, Charlottesville, Va.

Comparative studies of many new antibiotic analogues have placed considerable emphasis upon the relative concentrations achieved in blood and on *in vitro* determinations of minimum inhibitory concentrations against specific pathogens. Such assays have been necessary because of the many difficulties inherent in performing properly designed clinical comparisons of relative therapeutic efficacy. However, conventional cup-plate assays designed to measure total antibiotic serum concentrations are somewhat deceptive in that they provide no indication of that proportion of drug which is freely diffusable. Furthermore, it is reasonable to expect that a drug which is highly bound to plasma proteins will tend to be retained to a greater extent in the intravascular compartment than one which is highly diffusable, thereby achieving higher serum concentrations, (assuming all other factors governing distribution to be equal). In certain cases, excessive serum protein binding has rendered useless otherwise promising antibiotics(1,2); serum protein binding has been shown to be of considerable importance in the pharmacology of sulfonamides(3), the penicillins(1, 2,4,5) and tetracyclines(6,7,8).

Oral absorption studies provide only limited information concerning the effect of serum protein binding on blood concentrations, and ordinarily do not give adequate information regarding the removal of drug from the blood or its clearance by the kidneys. The present study was performed to delineate the effect of the known variations in serum protein binding(5,9-11) of 4 penicillin analogues on the concentrations achieved in the blood following intravenous injection in man and on the serum decay rates and urinary recoveries following this route of administration.

* Aided in part by grants from Wyeth Laboratories, Inc. and the Eli Lilly Co., Inc.

Materials and methods. Serum protein binding determinations were performed by suspending cellophane bags† containing 5 ml of pooled human serum in 10 ml of Krebs-Ringer phosphate buffer, pH 7.4, with 5, 10 or 20 units or μg of the appropriate drug. Dialysis was carried out for 24 hours at 8°C in a slowly rotating drum. Control bags contained 5 ml of buffer suspended in 10 ml of the various antibiotic solutions in buffer. Serum and buffer were removed at completion of incubation, stored at –20°C, and assayed as indicated below. All experiments were conducted in duplicate.

An intravenous dose of 500 mg of potassium benzyl penicillin‡ (G), phenoxymethyl penicillin‡ (V), phenoxyethyl penicillin‡ (Phenethicillin), (Ph) and sodium phenylmercaptomethyl penicillin§ (PM) was rapidly injected, in rotation, into 5 healthy young men. Doses were separated by at least one week. Venous blood samples were obtained at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 hours after the dose. Serums were separated as soon as possible and stored at –20°C until time of assay. All urine passed during 24 hours following the dose was collected in sterile containers and immediately refrigerated; aliquots from pools obtained at 0-1, 1-2, 2-3, 3-4, 4-8, and 8-24 hours were kept frozen at –20°C until time of assay. Specimens of serum and urine were assayed by the cup-plate method employing *Sarcina lutea* as the test organism and levels expressed in μg/ml based on comparison with standard drugs. These tests were done in the Research Laboratories, Wyeth Co. (to be referred to as Laboratory A) and the Eli Lilly Co. (Laboratory B) to which the specimens were

† Obtained from the Visking Co., Chicago, Ill.

‡ Kindly supplied with assay potency by Wyeth Laboratories.

§ Kindly supplied with assay potency by Eli Lilly Labs.

TABLE I. Human Serum Protein Binding of 4 Penicillin Analogues.

Analogue	Concentration/ml Pre-dialysis buffer	Post-dialysis Serum	Post-dialysis Buffer	% bound ± S.E.
Benzyl (G)	5 units	7.0	3.6	49.0
	"	6.3	3.3	48.3
	10	13.3	6.5	51.3
	"	10.9	5.7	47.9
	20	23.1	10.7	53.6
	"	29.7	17.1	42.3
Mean				48.7 ± 1.6
Phenoxymethyl (V)	5	6.9	1.5	78.2
	"	6.2	1.5	75.9
	10	15.9	4.7	71.7
	"	16.0	4.7	70.4
	20	31.3	7.6	75.7
	"	26.0	6.6	74.7
Mean				74.4 ± 1.2
Phenoxyethyl (phenethicillin) (Ph)	5	5.3	1.6	68.9
	"	5.6	1.4	75.0
	10	9.6	1.9	80.2
	"	9.8	1.9	81.1
	20	24.4	3.6	85.5
	"	26.3	4.6	82.5
Mean				78.8 ± 2.4
Phenylmercaptomethyl (PM)	5 μg	7.3	2.0	72.6
	" "	7.0	1.6	77.1
	10 "	20.1	3.2	84.1
	" "	15.1	3.0	80.1
	20 "	32.5	6.5	80.0
	" "	26.9	6.5	75.8
Mean				78.3 ± 1.6

delivered in the frozen state. All assays were performed on coded specimens. Laboratory A assayed concentrations of G, V and Ph in terms of each analogue while Laboratory B assayed concentrations of identical specimens of G and of PM in the same manner. Codes were not broken until results were reported to this laboratory.

Analyses of protein binding, half-life, relative distribution volumes, and tests of significance were conducted as previously reported (7).

Results. Serum binding. Serum protein binding data are summarized in Table I. Control tubes containing buffer in both bath and bag were found to have equilibrated within 24 hours. Concentrations of antibiotic pairs were either identical or within ± 10% of each other. Penicillin G was found to be the least bound to human serum (49%, differing significantly from all of the other analogues tested by 25-30%. Differences of binding between any 2 of the other analogues were not significant ($P = > .05$).

Serum levels. The average concentrations of penicillin G, V and phenethicillin in the serum of 5 subjects following an intravenous injection of 500 mg of each are presented in Fig. 1. Serum concentrations of penicillin G were somewhat lower than the other 2, but these differences are not significant. A linear relationship between the logarithm of the serum concentration *vs* time was demonstrated for each decay curve for the period of 1.5 through 4 hours following injection. Serum half-life and the y intercept at zero time were calculated by use of a regression equation(7) for each observation employing data obtained during that time period. Relative distribution volume (RDV) in liters and RDV per kg body weight in liters per kg were then calculated. These data are presented in Table II. Considerable variation was encountered among individuals and between the two different laboratories which assayed replicate samples of penicillin G in

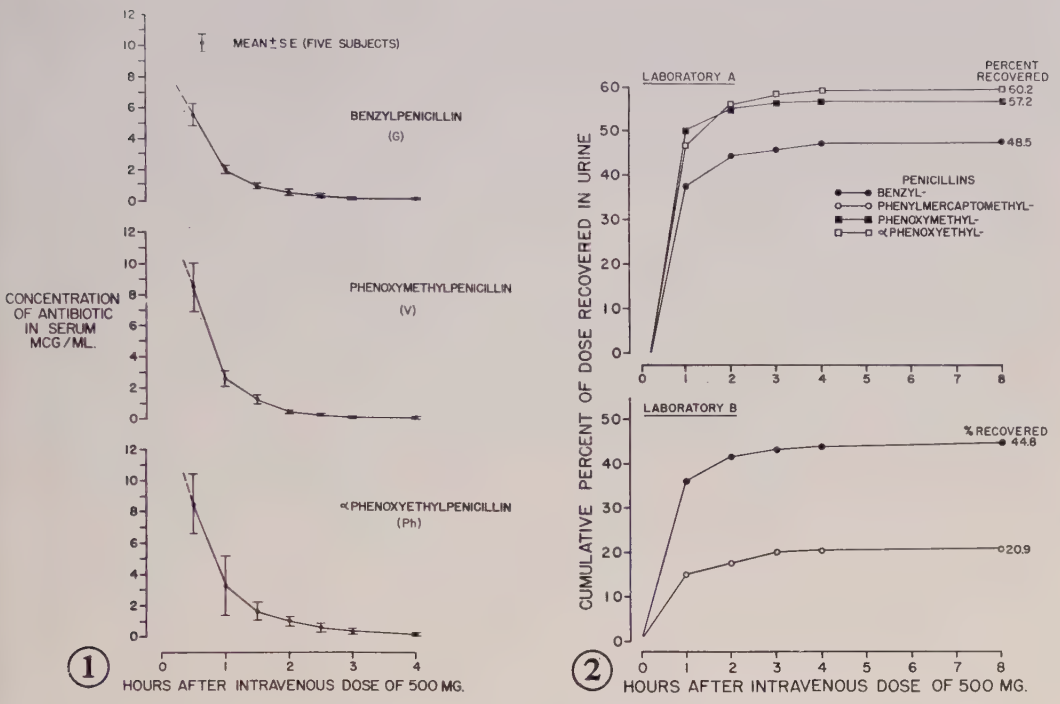


FIG. 1. Average serum concentrations of 3 penicillin analogues following intravenous injection of 500 mg of each in healthy young men (determined in Lab. A).

FIG. 2. Average cumulative 24 hr recoveries of 4 penicillin analogues following intravenous injection of 500 mg of each in healthy young men.

TABLE II. Relative Volume Distribution (RVD) of 4 Penicillin Analogues after Single Intravenous Injection.

	Lab. A				Lab. B			
Subject	Analogue	Co,† μg/ml	RVD, l	RVD/kg, l/kg	Analogue	Co, μg/ml	RVD, l	RVD/kg, l/kg
A	G*	2.6	192	2.5	G	23.3	21	.3
B		6.6	86	.9		8.1	62	.7
C		3.8	132	1.8		9.7	52	.7
D		5.8	86	1.5		4.3	116	2.0
E		1.7	294	3.8		9.6	52	.7
	Mean	4.1	156	2.1	Mean	11.0	61	.9
A	V	5.8	86	1.1	PM	6.3	79	1.0
B		4.9	102	1.3		5.8	86	1.0
C		8.3	60	.8		3.5	143	1.9
D		13.4	37	.6		9.0	56	1.0
E		7.9	63	.8		4.3	116	1.5
	Mean	8.1	70	.9	Mean	5.8	96	1.3
A	Ph	5.2	96	1.3				
B		4.7	106	1.3				
C		10.6	47	.6				
D		4.1	122	2.1				
E		5.0	100	1.3				
	Mean	5.9	94	1.3				

* The probability that the observed differences between any 2 of the above groups is due to chance is greater than .05.

† Calculated y intercept at zero time.

TABLE III. Half-Life of 4 Penicillin Analogues in Serum after Single Intravenous Injections.

Subject	Lab. A			Lab. B	
	G	V	Ph	G	PM
	Hr				
A	.68	.63	.65	.48	.57
B	.66	.53	.71	.79	.64
C	.70	.50	.66	.70	.65
D	.79	.48	1.03	1.14	.73
E	1.07	.49	.80	.64	.64
Mean ± S.E.	.78 ±.15	.52 ±.03	.77 ±.13	.75 ±.11	.64 ±.02

serum. The differences encountered between any 2 groups of data were not significant.

Much more uniform results were obtained for the half-life determinations (Table III). The half-life of penicillin G calculated from data provided by assays done in both laboratories was 0.78 and 0.75 hour, respectively, and was almost identical to that of phenethicillin at 0.77 hour. The half-life of penicillin V of 0.52 hour however was significantly lower ($P = <.001$) than all the others; phenylmercaptomethyl penicillin was intermediate at 0.64 hour, a half-life significantly different from G, V and phenethicillin.

Recovery in urine. Cumulative 24 hour urinary recoveries of the 4 analogues are presented in Fig. 2. Eighty to 90% of the total amount excreted of each of the analogues was recovered in the first 1-2 hours after injection. Almost identical amounts of penicillin G (48.5 and 44.8% respectively) were recovered by each of the 2 laboratories from replicate samples. Phenethicillin was most completely recovered (60.2%), closely followed by penicillin V (57.2%). The smallest recovery was noted with mercaptomethyl penicillin, only about 21% of which was found in the urine.

Discussion. The human serum protein binding of penicillin V and G reported here is almost identical to that measured by Smith *et al.*(9); the values for penicillin G are similar to those obtained by others(5,10,12). Pindell *et al.*(11), working with relatively large amounts of these 2 analogues (mg quantities) in canine serum, found binding of each to be only about one-half that noted by others, including ourselves, in human serum. In the present study phenethicillin and phenylmercaptomethyl penicillin were bound to about the same extent as penicillin V (70-80%) while only 49% serum binding was found with penicillin G.

No significant differences in relative distribution volume per kg body weight could be demonstrated among the analogues given intravenously to human volunteers. This was in part due to the considerable individual variations encountered between subjects and laboratories performing the assays. It would be premature to conclude from these limited observations that differences in distribution volume do not exist, but it is unlikely that the differences are greater than one-fold. Cronk and Wheatly(13), similarly, were unable to demonstrate significant differences in serum concentrations of penicillins G, V and phenethicillin following intramuscular injection in man. Holland *et al.*(14), however, concluded on the basis of intramuscular injection studies in a large group of human volunteers that the distribution volume of penicillin V was considerably greater than G, because of their finding of significantly higher serum concentrations achieved with the latter drug. It is possible that the results of these authors were due to incomplete absorption of penicillin V or some other factor related to the assay of this analogue, since the recovery of penicillin V in urine was only one-half of that found in the present study while the recovery of penicillin G was in accord with our observations and those of McCarthy and Finland(15).

All 4 penicillin analogues were rapidly cleared from serum following intravenous injection, the half-life in serum of penicillin G and phenethicillin were virtually identical; phenylmercaptomethyl penicillin was removed somewhat more rapidly; penicillin V was the most rapidly cleared analogue. Holland *et al.*(14) also noted a more rapid blood clearance of V than G.

The recovery of the 4 analogues in the urine following an intravenous injection reported here parallel the data that McCarthy and Finland obtained after giving an oral dose. The recoveries of penicillin V and phenethicillin over the same time period were about twice as great following an intravenous

dose than following an oral dose, indicating incomplete oral absorption of these analogues. It is of some interest that only 21% of the phenylmercaptomethyl analogue was recovered following an intravenous injection in the present study, whereas 18% was recovered in the urine by McCarthy and Finland following an oral dose. This comparison suggests that considerably more phenylmercaptomethyl penicillin was absorbed in their studies than would appear from examination of the serum or urine concentrations alone after an oral dose. This is of particular interest in view of the marked antimicrobial activity of this analogue(15,16). Employing the same considerations urinary recovery of penicillin G following oral administration is only about one-fifth that for parenterally administered drug(15).

When the effect of serum binding is considered in relation to serum concentrations achieved by the 4 analogues, it would appear that, on a weight basis, about 25-30% more free penicillin is available with penicillin G than the other analogues. This may be of some importance in considering the relative merits of these drugs for parenteral use, but in view of their much superior intestinal absorption this advantage of penicillin G is readily overcome by the other analogues when used orally.

Many additional considerations must be taken into account in the choice of a particular analogue for a particular therapeutic task, including activity weight for weight against different organisms, cost, optimal dosage and completeness of absorption.

Summary.. The *in vitro* binding to human serum proteins, and the half-life in serum, relative volume distribution and urinary recovery of 4 penicillin analogues, G, V, phenethicillin, and mercaptomethyl penicillin following single intravenous injections in normal male subjects were investigated to aid interpretation of oral absorption studies. Penicillin G was found to be significantly less bound than the others (48.7% compared to 74.4 – 78.8%). Significant differences in relative distribution volumes of the 4 analogues could not be demonstrated due in large part to considerable subject and laboratory variation. The shortest half-life in serum was obtained with penicillin V (0.52 hour), the longest with penicillin G and phenethicillin (0.75-0.78 hour), while phenylmercaptomethyl penicillin was intermediate (0.64 hour). The 24-hour urinary recoveries were penicillin G 46%, V 57%, phenethicillin 60%, and phenylmercaptomethyl penicillin 21%. It is concluded from comparison of these results with those of others that differences noted in protein binding may be outweighed by other considerations such as cost, optimum therapeutic dose, relative antimicrobial activity and extent of gastro-intestinal absorption when oral preparations are used.

1. Hobby, G. L., Burkhart, B., Hyman, B., PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 296.

2. Eagle, H., *J. Exp. Med.*, 1947, v85, 175.

3. Davis, B. D., *J. Clin. Invest.*, 1943, v22, 753.

4. Tompsett, R., Shultz, S., McDermott, W., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 163.

5. ———, *J. Bact.*, 1947, v53, 581.

6. Sirota, J. H., Saltzman, A., *J. Pharmacol. & Exp. Therap.*, 1950, v100, 210.

7. Kunin, C. M., Dornbush, A. C., Finland, M., *J. Clin. Invest.*, 1959, v38, 1950.

8. Roberts, C. E., Jr., Perry, D. M., Kuharic, H. A., Kirby, W. M. M., *Arch. Int. Med.*, 1961, v107, 204.

9. Smith, C. I., Levin, J. D., Embody, D. R., In: *Antibiotics Annual,* New York, N Y., Medical Encyclopedia, Inc., 1957, p306.

10. Anderson, R. C., Lee, C. C., Worth, H. M., Chen, K. K., *ibid.*, 1956, p540.

11. Pindell, M. H., Tisch, D. E., Hoekstra, J. B., Reiffenstein, J. C., In: *Antibiotics, Annual,* New York, N. Y., Antibiotica, Inc., 1960, p119.

12. Chow, B. F., McKee, C. M., *Science,* 1945, v101, 67.

13. Cronk, G. A., Wheatley, W. B., *Am. J. Med. Sci.*, 1960, v240, 722.

14. Holland, S. M., Livesey, E. F., Owen, J., Pennington, G. W., Roberts, J. B., Somers, G. F., *Antibiot. and Chemother.*, 1960, v10, 25.

15. McCarthy, C. G., Finland, M., *New Eng. J. Med.*, 1960, v263, 315.

16. McCarthy, C. G., Wallmark, G., Finland, M., *Am. J. Med. Sci.*, 1961, v241, 143.

Received April 6, 1961. P.S.E.B.M., 1961, v107.

Direct Humoral Control of Parathyroid Function in the Dog.*† (26619)

D. Harold Copp and A. George F. Davidson
Department of Physiology, University of British Columbia, Vancouver, Canada

The primary function of the parathyroids is the control of blood calcium(1,2). It is generally agreed that this control is reciprocal. Rasmussen(3) states that: "the primary factor regulating the secretory activity of the parathyroid glands is the calcium ion activity (A_{Ca++}) of the plasma." However, evidence for this has been largely indirect. Ham *et al.*(4) demonstrated hyperplasia of the parathyroids of calcium depleted rats. Stoerk and Carnes(5) showed that this enlargement correlated directly with the lowered serum calcium values of these animals, but had no definite relation to the inorganic phosphate level in the plasma. Toft and Talmage(6) have observed a quantitative increase in numbers of osteoclasts in the long bones of rats associated with the hypocalcemia produced by continuous peritoneal lavage with calcium-free rinse. Patt and Luckhardt(7) perfused calcium depleted blood through an isolated dog thyroid-parathyroid gland, and when this perfusate was subsequently injected into a second dog, they demonstrated a rise in blood calcium similar to that obtained with parathyroid extract. Their experiments provided the stimulus for the studies reported here in which a direct humoral control of parathyroid function has been demonstrated.

Procedures. Dogs were fasted overnight and anesthetized with nembutal, the right thyroid-parathyroid glands were removed, and the left glands were exposed, along with the carotid and superior thyroid arteries. The muscular branches of the latter were ligated. A dose of 0.5 mg/kg heparin was then injected *i.v.* to prevent clotting in the apparatus, and the carotid was cannulated proximally. Blood from the cannula was conducted in plastic tubing through a variable speed perfusion pump (Model TM-11, Sigmamotor, Inc., Middleport, N. Y.) to a second cannula inserted into the carotid well below the superior thyroid artery and directed towards it. The carotid was then tied above this branch, so that blood from the pump flowed only through the thyroid and parathyroids supplied by it. The tubing entering and leaving the animal was bound together in an insulated sheath, so that the returning blood was warmed by counter-current exchange. The experimental arrangement is shown diagrammatically in Fig. 1. Systemic blood pressure and perfusion pressure were observed directly.

The plasma calcium in the perfusing blood was raised approximately 4 mg % (1 mM/l) by addition of a solution containing 5 mM/l calcium chloride or calcium gluconate, or was lowered by addition of an equivalent amount of the calcium complexing agent disodiumethylenediaminetetraacetate (EDTA). Both were made up in physiological saline containing 50 mg/l heparin. They were pumped through tubes with approximately 10% of the capacity of the blood

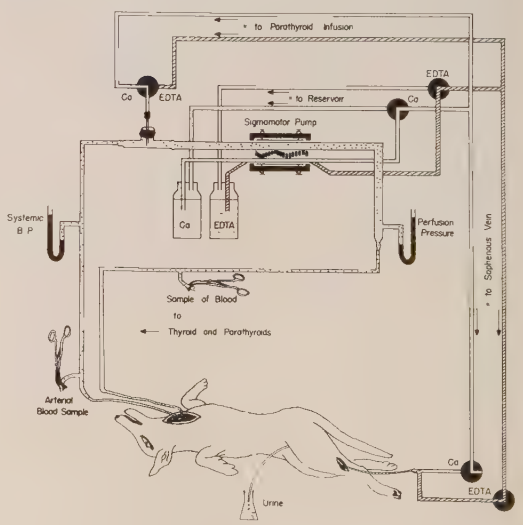


FIG. 1. Apparatus for perfusion of thyroid-parathyroid glands of the dog *in situ*.

* Aided by grants from Division of Med. Research, Nat. Research Council, Canada. Parathyroid extract supplied through courtesy of Eli Lilly & Co.

† The authors wish to acknowledge the invaluable technical assistance of B. Cheney, L. Ker, K. Henze and J. Dubé.

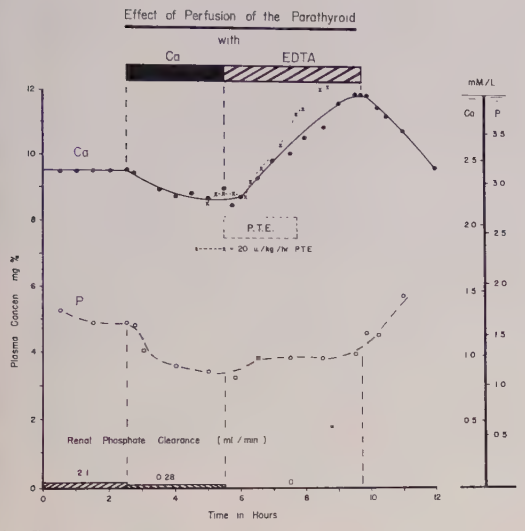


FIG. 2. Typical response of systemic plasma calcium and inorganic phosphate to perfusion of parathyroid with blood to which calcium or EDTA had been added. Dotted line shows changes in the same dog when parathyroid extract (20 u./kg/hr) was injected for a 3 hour period 6 hours after parathyroidectomy.

tube, using the same pump. In this way, the proportion of calcium or EDTA added to the blood remained constant despite the variations in pump speed which were required to maintain a perfusion pressure of 120-150 mm Hg. The flow through the thyroid-parathyroids varied from 5-20 ml/min. By using 3 way stopcocks, it was possible to direct Ca or EDTA into the saphenous vein, the blood perfusing the parathyroids, or the appropriate reservoir. During the control period (2 hr), both Ca and EDTA were perfused into the saphenous vein, and since they form a physiologically inert complex, there was essentially no change in calcium balance. Calcium was then switched to the parathyroid perfusate, while EDTA continued to flow into the saphenous vein. The animal remained in calcium balance, but the parathyroids were exposed to hypercalcemic blood. After 2-3 hours, the solutions were reversed, so that the parathyroids were exposed to blood low in calcium.

Samples of systemic arterial blood and parathyroid perfusion blood were collected at intervals of 15-30 minutes. Plasma calcium was determined by potentiometric titration with EDTA, using a modification of the method of Lehmann(8). Inorganic phosphate in plasma and urine was determined by the Taussky and Shorr(9) modification of the method of Fiske and Subbarow. Renal clearance of phosphate was determined over 2 hr clearance periods. Fifteen animals were studied.

Experimental results. A typical experiment is illustrated in Fig. 2. Plasma calcium remained constant during the control period. Shortly after beginning perfusion of the parathyroids with high calcium blood, there was a fall in systemic blood calcium which began to level off after 2 hours. When the glands were now perfused with low calcium blood, there was a delay of 30 minutes, followed by a sharp rise in blood calcium, reaching a level of 12 mg% within 4 hours. Perfusion of the gland was now stopped, and continuity of the carotid was restored. Almost immediately, blood calcium fell towards normal. Two days later, the animal was normocalcemic. The surviving parathyroid and the arterial graft were now removed, and 6 hours later, the animal received a continuous *i.v.* infusion of 20 units Parathyroid Extract (Lilly) per kg/hr for 3 hours. As shown by the dotted curve in Fig. 2, there was a rise in plasma calcium which corresponded to that obtained with hypocalcemic stimulation of the parathyroid.

There was no significant change in plasma inorganic phosphate during stimulation of the gland with low calcium blood. This was observed in 8 perfusions; in 4 the plasma phosphate rose during this phase; in 3 there was a fall associated with increased excretion of phosphate in the urine. In one experiment, illustrated in Fig. 3, inorganic phosphate was injected *i.v.* during this phase, doubling plasma phosphate concentration without preventing the rise in plasma calcium.

Discussion. These experiments show that the level of calcium in the blood perfusing the parathyroids has a direct effect on their function with respect to regulating blood calcium, and provide a basis for the "feedback" mechanism proposed by McLean(2). The effects are sufficiently sensitive and fast-acting to explain the acute homeostatic con-

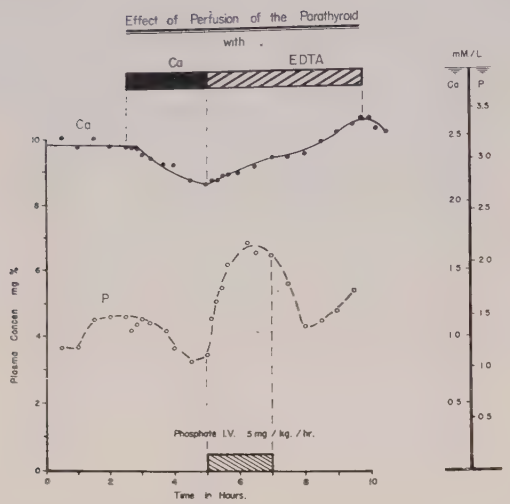


FIG. 3. Changes in plasma calcium and inorganic phosphate in a dog during perfusion of the parathyroids with high calcium (Ca) or low calcium (EDTA) blood. During the phase of hypocalcemic stimulation of the gland, isotonic phosphate solution (5 mg P/kg/hr) was injected *i.v.* for 2 hours.

trol of calcium in body fluids(10). Perfusion of the glands with low calcium blood caused a rise in systemic plasma calcium similar to that resulting from continuous *i.v.* infusion of parathyroid extract, so that this effect is probably due to liberation of parathyroid hormone.

Albright(11) has suggested that the rise in serum calcium resulting from injection of parathyroid hormone is secondary to a fall in blood phosphate. This was certainly not the case in our experiments. During this phase of "physiological stimulation" of parathyroid hormone production, the rise in plasma calcium occurred despite the fact that in most instances the blood phosphate remained unchanged or even increased. Patt and Luckhardt(12) also observed a rise in blood phosphate associated with the rise in calcium following injection of their parathyroid perfusate.

Summary. Direct humoral control of parathyroid function has been demonstrated in dogs in which the isolated thyroid-parathyroid glands were perfused with high calcium or low calcium blood. The latter appears to release parathyroid hormone or a substance with identical action. The resulting rise in systemic blood calcium does not depend on a fall in the inorganic phosphate of blood. These experiments indicate a "feedback" mechanism involving the parathyroids which is sufficiently sensitive and fast-acting to account for the acute homeostatic control of blood calcium.

1. Howard, J. E., Connor, T. B., *Bibliot. Paediat.,* (Suppl.), 1954, v58, 230.

2. McLean, F. C., *Clin. Orthopaed.,* 1957, v9, 46.

3. Rasmussen, H., *Am. J. Med.,* 1961, v30, 112.

4. Ham, A. W., Littner, N., Drake, T. G. H., Robertson, E. C., Tisdall, F. F., *Am. J. Path.,* 1940, v16, 277.

5. Stoerk, H. C., Carnes, W. H., *J. Nutrition,* 1945, v29, 43.

6. Toft, R. J., Talmage, R. V., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 611.

7. Patt, H. M., Wallerstein, E., Luckhardt, A. B., *ibid.,* 1942, v49, 580.

8. Lehmann, J., *Scandinav. J. Clin. and Lab. Invest.,* 1953, v5, 203.

9. Taussky, H. H., Shorr, E., *J. Biol. Chem.,* 1953, v202, 675.

10. Copp, D. H., Mensen, E. D., McPherson, G. D., *Clin. Orthopaed.,* 1960, v17, 288.

11. Albright, F., *J. Am. Med. Assn.,* 1941, v117, 527.

12. Patt, H. M., Luckhardt, A. B., *Endocrinology,* 1942, v31, 384.

Received April 7, 1961. P.S.E.B.M., 1961, v107.

In vitro RBC Uptake of Radio-Insulin: A Simple Method for Detecting Insulin Antibodies.* (26620)

JULIAN I. ZINDER,† SHELDON BERGER, AND M. S. GOLDSTEIN
Department of Metabolic and Endocrine Research, Michael Reese Hospital and Medical Center, Chicago, Ill.

The *in vitro* uptake of I^{131}-labelled thyroxine or triiodothyronine by the red blood cell (RBC) is being employed for diagnostic and investigative purposes in relation to thyroid function. The concept underlying this technic presumes that *in vitro* distribution of thyroid hormones between RBCs and plasma is a function of the competitive binding affinities of the RBCs and certain of the plasma proteins. It may be possible to view the *in vitro* red blood cell uptake as reflective of hormone availability to the peripheral tissues. Substituting I^{131} insulin for I^{131} thyronines in a similar *in vitro* method, a study was undertaken of the insulin I^{131} partition between plasma and RBCs of normal and diabetic subjects, some of whom were, or had been, treated with insulin.

Method. Patients from Medical and Surgical services of the Outpatient Clinics, Michael Reese Hospital, served as subjects. Most determinations were made within 2 hours of collecting the blood specimens in heparinized tubes. Several specimens were examined 24 hours after storage in the cold with similar results.

The essential method and calculations are those previously described for the red blood cell uptake of I^{131} triiodothyronine(1) with the exception of using I^{131} insulin instead of I^{131} triiodothyronine. I^{131} insulin was added to 3 ml aliquots of whole blood in a stoppered, 10 ml Erlenmeyer flask and incubated, with shaking, at 37°C for 2 hrs. Radioactive insulin of a specific activity of 4.7-6.5 μc/mg was utilized, and 1×10^{-6} to 2.5×10^{-4} mg of insulin was added per flask. The radioactive content of two 1 ml aliquots of whole blood (at counting rates sufficient to permit 5% accuracy) was determined in a well type scintillation counter. The radioactivity of the RBC mass in each 1 ml aliquot of whole blood was determined after 5 washings in 10-fold dilutions of isotonic sodium chloride and centrifugation at 3,000 RPM for 10 minutes to separate cells from plasma or saline wash. The percent RBC uptake was calculated by the formula:

$$\% \text{ RBC uptake} = \frac{\text{net counts in RBC} \times 100 \times 100}{\text{net counts/ml of whole blood} \times \% \text{ hematocrit}}$$

This result yields an expression of RBC uptake for a hypothetical hematocrit of 100%, as previously described(1). "Criss-cross" experiments were performed by separating plasma and blood cells, washing the blood cells 3 times with isotonic saline, then resuspending normal cells in diabetic plasma and *vice versa*.

The influence of the white blood cell count was tested by centrifuging heparinized blood samples at 3,000 RPM for 10-15 minutes. The "buffy coat", adjacent cell layers and a proportionate amount of plasma were removed so as to preserve the original hematocrit. Each original sample accordingly yielded a white cell depleted and white cell enriched sample. White blood cells were counted in a conventional clinical chamber.

Results. Both single and multiple determinations were carired out in 113 adult patients. Of these, 37 subjects were non-diabetics who had never received insulin; 10 patients were diabetics managed with diet alone; 24 subjects were diabetics treated with oral sulfonylureas alone; 47 patients were diabetics having received insulin for variable periods of time in excess of 3 months and at variable dose schedules; and 8 patients had previously received chronic insulin therapy but had been without insulin from one month to 14 years.

Table I indicates that there is a distinct

* Supported by grant from Nat. Inst. of Arthritis and Metab. Dis.

† Trainee, Nat. Inst. Health.

TABLE I. Uptake of Radioactive Insulin by RBC of Normal and Diabetic Subjects: Influence of Various Forms of Treatment.

Condition	Treatment	No.	% radio-insulin uptake by RBC*
Normal	—	47	6.2 ± .4
Diabetic	Diet alone	8	5.1 ± .7
"	Sulfonylureas alone	24	5.7 ± .4
"	Insulin for at least 3 mo	36	2.3 ± .3

* ± stand. dev.

reduction in uptake of radio-insulin by the RBC of the diabetic group receiving insulin currently as compared with both non-diabetics and diabetics treated by means other than exogenous insulin. No significant distinction can be made between non-diabetics and non-insulin treated diabetics. There was no correlation between degree of radio-insulin uptake by RBC with dosage of insulin administered or with duration of prior treatment in excess of 3 months.

After cessation of insulin therapy, the reduced radio-insulin uptake by RBC may persist for as long as 4 years or return to normal values within one year. Table II indicates the wide spread of findings, from 1.3% to 11.2% in 8 such patients whose insulin therapy was interrupted for variable periods of time.

The reduced uptake of radio-insulin by the RBC of insulin-treated diabetics is a function of the plasma and not the RBCs (Table III). So called "criss-cross" experiments in which plasma and cells were exchanged between a diabetic subject and a type-compatible normal indicate that the cause for the reduced RBC uptake is resident in the plasma of the insulin treated individual and is not influenced by any intrinsic property of the RBC.

TABLE II. Effect of Elapsed Time Since Interruption of Insulin Therapy on the Reduced Uptake of Radio-Insulin by RBC of Diabetic Subjects.

Patient	Elapsed time since insulin treatment	% radio-insulin uptake by RBC
1	1 mo	2.6
2	2 "	1.3
3	1 yr	11.2
4	3 "	2.2
5	3 "	6.5
6	3 "	4.7
7	4 "	2.6
8	14 "	3.8

Since a marked fixation of I^{131} insulin has been described for leucocytes in peritoneal exudates(2) of rabbits, the possible influence of white blood cells was examined. Artificially varying the white cell count of the same blood sample 4-5 fold does not reflect itself in any change in blood cell uptake (Table IV). It appears that circulating human leucocytes do not exhibit appreciable uptake of radioactive insulin in this technic.

TABLE III. Importance of Plasma in the Reduced Uptake of Radio-Insulin by RBC after Insulin Therapy As Seen in "Criss-Cross" of Normal and Diabetic RBC and Plasma.

Plasma + RBC	No.	% radio-insulin uptake by RBC*
Normal + Normal	6	7.4 ± 3.9
Diabetic + Diabetic	6	3.2 ± 1.4
Normal + Diabetic	6	6.5 ± 2.1
Diabetic + Normal	6	3.1 ± 1.4

* ± stand. dev.

Discussion. Berson and Yallow assayed the radioactivity of packed, unwashed erythrocytes following an intravenous injection of I^{131} labelled insulin. Erythrocyte radioactivity paralleled non-trichloracetic acid precipitable radioactivity. The erythrocyte radioactivity could represent insulin free of plasma binding protein or some *in vivo* breakdown product. In insulin-treated subjects, erythrocyte radioactivity appeared at a slower rate and to a lesser extent than in non-insulin treated subjects. This suggested increased binding of insulin in the plasma of the insulin-treated group and less available

TABLE IV. Lack of Influence of Leucocytes in Determining Radio-Insulin Uptake by Blood Cells: Artificial Enrichment and Depletion of Leucocytes in the Same Blood Sample.

Subject	Leucocytes/mm³	% radio-insulin uptake by RBC
1	Enriched 9,400	3.7
	Depleted 2,200	4.1
2	Enriched 6,700	2.8
	Depleted 1,200	2.4
3	Enriched 11,000	6.3
	Depleted 2,800	6.3

insulin for the erythrocytes. By a number of technics, this change in blood cell-plasma partition was demonstrated to be attributable to an insulin-binding globulin induced only in insulin-treated subjects, irrespective of their diabetic status. These technics included paper strip chromato-electrophoresis, starch block electrophoresis, ultra-centrifugation of insulin I^{131}-plasma mixtures, precipitation of insulin antibody complexes with rabbit anti-human globulin serum, etc.(3,4).

By use of a relatively simple technic, we believe that we have shown the presence of an insulin-binding plasma factor in subjects who have received insulin. We have not attempted qualitatively to correlate this with determinations done by other technics. However, because this plasma binding factor was insulin-induced and, as others have found (3,4), did not quantitatively correlate with either dosage or duration of insulin therapy, this procedure appears to demonstrate the presence of the reported insulin-binding globulin. In these studies the reduced RBC uptake appeared between the third week and third month of the institution of insulin treatment; it persisted for longer than 2 months and, in one patient, for 4 years after insulin treatment had been discontinued. This time relationship is consistent with the appearance and disappearance of the insulin antibody as determined by other technics(3, 4).

It must be noted that the present method cannot distinguish between red blood cells and leucocytes. However, we have demonstrated that the leucocyte contribution to the blood cell uptake may be regarded as unimportant in this technic.

Summary. The capacity of RBC to take up radioactive insulin (I^{131}) from whole blood has been studied in blood samples from normal and diabetic subjects. Prior insulin therapy induces a marked reduction in RBC uptake. Dietary management and/or sulfonylurea therapy does not induce a reduced RBC uptake of radio-insulin. These findings are interpreted to represent a simple technic for demonstrating the presence of insulin antibodies.

1. Hamolsky, M. W., Golodetz, A., Freedberg, A. S., *J. Clin. Endocrinol. and Metab.*, 1959, v19, 103.
2. Haugaard, N., Haugaard, E. S., Stadie, W., *J. Biol. Chem.*, 1954, v211, 289.
3. Berson, S. E., Yallow, R. C., *J. Clin. Invest.*, 1956, v35, 170.
4. Skom, J. H., Talmage, D. W., *ibid.*, 1958, v37, 787.

Received April 7, 1961. P.S.E.B.M., 1961, v107.

Effect of Triparanol (MER/29) on Corticosterone Secretion by Rat Adrenals.* (26621)

JOHN HOLLOSZY AND ALBERT B. EISENSTEIN
Jewish Hospital of Saint Louis and Departments of Medicine and Preventive Medicine, Washington University School of Medicine, St. Louis, Mo.

Triparanol (MER/29) is used as an agent to lower serum cholesterol. Studies by Avigan, Steinberg and coworkers suggest that triparanol inhibits conversion of desmosterol (24-dehydrocholesterol) to cholesterol, the final step of cholesterol synthesis(1). There is also evidence which shows that the total sterol pool is significantly decreased during triparanol therapy(2). Blohm, Kariya and Laughlin(3) have demonstrated that feeding triparanol to rats results in a marked reduction of adrenal cholesterol. Since cholesterol is an important precursor of adrenocortical hormones, it was thought that triparanol feeding might also result in decreased steroid hormone synthesis by this gland. For this reason the present study was initiated.

Materials and methods. Young, male rats

* Supported by grants from Nat. Vitamin Foundation and Nat. Inst. of Arthritis and Metab. Dis.

TABLE I. Effect of Triparanol Feeding on Adrenal Function in Rats in Exp. I.

	Left adrenal wt (mg)	Adrenal cholesterol content (mg)	Adrenal vein plasma flow (ml/hr)	Plasma corticosterone (γ/ml)	Corticosterone secretion rate (γ/hr)
Triparanol treated rats (10)	24 ± .75*	2.55 ± .16	4.39 ± .45	12.8 ± .83	56.3 ± 6.4
Control rats (13)	20 ± .39	8.80 ±1.002	4.33 ± .44	17.5 ±1.39	71.4 ± 5.7

* Mean values ± stand. error of mean.

of the Holtzman strain were used. Rats were maintained on a diet of Purina laboratory chow and water *ad lib*. Triparanol was suspended in a carboxymethyl cellulose vehicle and fed to the animals by stomach tube.

Two experiments were conducted. In Experiment I, 15 rats, average initial weight 182 g, were fed 50 mg of triparanol per kg body weight for 13 days and 30 mg/kg for the next 12 days. The dose was reduced after 13 days because animals receiving triparanol were not gaining weight as rapidly as controls. Fifteen control rats, average initial weight 181 g, were given an equal volume of carboxymethyl cellulose for 25 days.

In Exp. II, 13 rats, average initial weight 192 g, were given 30 mg of triparanol per kg body weight by stomach tube daily for 35 days. Twelve control rats, average initial body weight 188 g, were given the vehicle alone.

Following the period of triparanol feeding, rats were anesthetized with pentobarbitol given intraperitoneally and adrenal vein blood was collected by the method of Longwell(4). Adrenal vein blood was collected for 2 consecutive 10 minute periods and since the volumes of the 2 samples were essentially the same, the samples were pooled, centrifuged and plasma taken for corticosterone analysis. Duplicate hematocrit determinations were done on blood from each rat. Plasma corticosterone was determined by the method of Silber *et al.*(5). When blood collections were complete, rats were sacrificed, the adrenals removed, dissected free of surrounding tissue and weighed. Total adrenal cholesterol was determined by the method of Knobil *et al.*(6).

Results. Exp. I is summarized in Table I. Triparanol feeding at a dose level of 30 mg/kg did not affect appetite or rate of growth of rats since at the end of the study treated animals weighed an average of 279.3 g while average weight of controls was 284.3 g. Adrenal vein plasma flows and left adrenal weights were also essentially the same in the 2 groups. Total cholesterol content of left adrenals from triparanol treated rats was decreased to 29% that of controls ($P = < .001$). Adrenal vein plasma corticosterone concentration was significantly lower in the treated group than in control rats, ($P = <0.05$). Rate of corticosterone production was also depressed by triparanol; however, this reduction was not statistically significant since $P = >0.05$.

In the second experiment (Table II) the period of triparanol feeding was extended by 10 days. Final body weight of treated rats averaged 348.5 g, while body weight of controls averaged 356.7 g. As observed in Exp. I, adrenal weight and adrenal vein plasma flow were not affected by triparanol feeding at this dose level. Adrenal cholesterol fell to 27% of control values in response to triparanol ($P = <0.01$). Adrenal vein plasma corticosterone concentration and corticosterone secretion rate were both significantly depressed in the triparanol treated group ($P = <0.05$).

Discussion. A considerable body of data has been accumulated through the use of adrenal slices, homogenates and perfusion technics which indicates that cholesterol is an important precursor of the adrenal steroid hormones(7,8). Other evidence, however, suggests that the adrenals can synthesize steroids from acetate without cholesterol being formed as an intermediate(9). The significance of this alternate pathway of steroid biosynthesis or confirmation of its occurrence has not been established.

Since cholesterol is an important precursor

TABLE II. Effect of Triparanol Feeding on Adrenal Function in Rats in Exp. II.

	Left adrenal wt (mg)	Adrenal cholesterol content (mg)	Adrenal vein plasma flow (ml/hr)	Plasma corticosterone (γ/ml)	Corticosterone secretion rate (γ/hr)
Triparanol treated rats (9)	25.2 ± 1.2 *	3.7 ± .27	4.92 ± .50	9.85 ± .91	45.8 ± 3.3
Control rats (9)	23.4 ± .95	13.8 ±1.6	5 07 ± .74	16.54 ±2.6	76.3 ±10.1

* Mean values ± stand. error of mean.

of adrenal steroids, it seems probable that any agent which inhibits synthesis of this sterol will result in diminished production of adrenal steroids. This hypothesis is borne out by results of the study reported here in which triparanol feeding caused a significant lowering of both adrenal cholesterol and the amount of corticosterone secreted by rats subjected to the stress of surgery and bleeding. Similarly, Melby, St. Cyr and Dale have demonstrated a 45% reduction in urinary excretion of 17-hydroxycorticosteroids in response to ACTH stimulation following a short period of triparanol feeding in humans(10). These investigators also found that urinary aldosterone and plasma cortisol levels were decreased in patients treated with this agent.

The finding that triparanol feeding results in a decrease in adrenal steroid production may have clinical significance in patients taking this compound. It seems conceivable that such an individual might develop acute adrenal insufficiency when exposed to the stress of major surgery or severe infection if not given prophylactic steroid therapy.

Summary. Triparanol feeding produces a significant reduction in the amount of corticosterone secreted by the adrenals of rats subjected to surgery and bleeding. It is postulated that this reduction is secondary to the marked decrease in adrenal cholesterol which results from triparanol therapy.

1. Avigan, J., Steinberg, O., Thompson, M. J., Mosetig, E., Suppl. to *Prog. Cardiov. Dis.,* 1960, v2, 525.

2. Hollander, W., Chobanian, A. V., Wilkins, R. W., *J.A.M.A.,* 1960, v174, 5.

3. Blohm, T. R., Kariya, T., Laughlin, M. W., *Arch. Biochem. and Biophys.,* 1959, v85, 250.

4. Longwell, B. B., Reif, A. E., Hansburg, E., *Endocrinology,* 1958, v60, 641.

5. Silber, R. H., Busch, R. D., Oslapas, R., *Clin. Chem.,* 1958, v4, 278.

6. Knobil, E., Hagney, M. G., Wilder, E. J., Briggs, F. N., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 48.

7. Saba, N., Hechter, O., Stone, D., *Arch. Biochem.,* 1955, v58, 249.

8. Hechter, O., Pincus, G., *Physiol. Rev.,* 1954, v34, 459.

9. Heard, R. D. H., Bligh, E. G., Cann, M. C., Jellnick, P. H., O'Donnell, V. J., Rav, B. G., Webb, J. L., *Rec. Prog. Horm. Res.,* 1956, v12, 45.

10. Melby, J. C., St. Cyr, M., Dale, S. L., *New Engl. J. Med.,* 1961, v264, 583.

Received April 7, 1961. P.S.E.B.M., 1961, v107

Potassium Content of Rat Diaphragms Following Hypophysectomy and Incubation with Growth Hormone.* (26622)

V. S. CONSTANTINE AND J. L. KOSTYO

Departments of Physiology and Medicine and Division of Endocrinology, Duke University, Durham, N. C.

The transport of α-aminoisobutyric acid (AIB) by isolated rat diaphragms is decreased following hypophysectomy and stimulated by addition of various species of growth hormone to the incubation medium(1,

* Supported by contract from Medical Research and Development Division, Office of Surgeon General, Dept. of the Army.

2). Certain findings suggested that these alterations in amino acid transport might be related to changes in muscle K^+ content. Batts *et al.*(3) reported that the K^+ content of rat skeletal muscle was decreased after hypophysectomy and restored to normal by growth hormone administration. Moreover, the transport of amino acids into Ehrlich mouse ascites tumor cells was found to be related to the level of intracellular K^+(4-6). The present experiments were performed to determine if hypophysectomy and growth hormone influence amino acid transport by muscle through alterations in muscle K^+ content.

Methods. Normal and hypophysectomized female rats weighing 50-80 g were purchased from the Charles River Breeding Laboratories. The rats were hypophysectomized at 24-25 days of age and used for experimentation 2-4 weeks following operation. The animals were killed by cervical fracture. In some instances, the diaphragmatic muscle alone was removed from the rat, washed in Krebs bicarbonate buffer, blotted, weighed and digested in nitric acid in preparation for K^+ measurements. For *in vitro* studies, rib cage preparations of diaphragms were prepared according to the method of Kipnis and Cori(7). They were placed in 125 ml Erlenmeyer flasks containing 50 ml of Krebs bicarbonate buffer, pH 7.4, and 0.01 M glucose, gassed with 95% O_2-5% CO_2, and preincubated with shaking for 10 min at 37°C. Following preincubation they were transferred to flasks containing 30 ml of the same medium and incubated under similar conditions for various lengths of time. In some cases bovine growth hormone (NIH-BGH-1)† was added to the medium to a concentration of 25 μg/ml. At the end of the incubation period, the diaphragmatic muscle was dissected away from the rib cage, blotted, weighed and digested in nitric acid. K^+ determinations were made with a Baird flame photometer with lithium internal standard.

† We thank the Endocrinology Study Section, Nat. Inst. Health for bovine growth hormone (NIH-BGH-1).

One experiment was designed to indicate the degree of amino acid transport by diaphragms which were K^+ depleted *in vitro*. Eight rib cage preparations were placed in a 6 liter Erlenmeyer flask containing 5 liters of bicarbonate buffer, which had the composition of Krebs buffer except that all of the K^+ was replaced by Na^+. Glucose was added to a concentration of 0.01 M. The muscles were incubated in this medium at 37°C with gentle stirring and continuous gassing with 95% O_2-5% CO_2 for 2 hours. This procedure resulted in a 35-40% loss in K^+ by the diaphragm. Following K^+ depletion, the diaphragms were blotted lightly on filter paper and transferred to 50 ml Erlenmeyer flasks containing 10 ml of the K^+-free bicarbonate buffer, 0.01 M glucose and 0.05 mM AIB-1C^{14} (S.A. = 1 mc/mM). The flasks were gassed with 95% O_2-5% CO_2, sealed and incubated with shaking at 37°C for 1 hour. After incubation, the AIB in the diaphragmatic muscle was extracted and counted by previously described methods(2). The results are expressed as the AIB distribution ratio which is the ratio of the cpm/ml of intracellular water to the cpm/ml of incubation medium. This distribution ratio gives an indication of the degree to which the cells concentrated the amino acid during the incubation period. The method used to calculate the radioactivity in the intracellular water has been described previously (*ibid.*).

Results and discussion. Hypophysectomy did not alter the *in vivo* K^+ content of rat diaphragms (Table I). Consequently, the impairment in amino acid transport by diaphragm muscle caused by hypophysectomy cannot be related to a low *in vivo* muscle K^+ level. However, it had been reported previously that rat diaphragm loses large amounts of K^+ when incubated *in vitro* under simulated physiological conditions(8,9,10). This suggested that diaphragms of hypophysectomized rats might lose greater amounts of K^+ than those of normal rats during incubation *in vitro*, resulting in a low K^+ level which would then be responsible for impaired amino acid transport. The results in Table I would indicate that this is not the case. Even after 2 hours of incubation *in vitro* there was no

TABLE I. Effects of Hypophysectomy and Incubation with Growth Hormone on K^+ Content of Rat Diaphragm.

Conditions	Muscle K^+ content (μeq/g wet wt)		
	Normal	Hypox.	Hypox. + G.H.
In vivo	101 ± 1.55*(11)	102 ± .81 (6)	
In vitro, 60 min. incubation†	103 ± 1.15 (5)	106 ± 6.27 (4)	106 ± 1.38 (4)
120 " "	102 ± 2.30 (4)	107 ± 1.38 (4)	110 ± 2.75 (4)

* Mean ± S.E. No. of animals in parentheses. † All diaphragms preincubated for 10 min.

difference in K^+ content between normal and hypophysectomized rat diaphragms. Moreover, when hypophysectomized rat diaphragms were incubated with bovine growth hormone, muscle K^+ content was not altered (Table I). Thus neither hypophysectomy nor incubation with growth hormone exerted a detectable influence on the K^+ content of rat diaphragms *in vitro*.

It was conceivable that hypophysectomy and incubation with growth hormone might produce changes in diaphragm K^+ content which were too small to be detected by the method used and yet large enough to alter significantly the rate of amino acid transport by the cells. Should growth hormone stimulate amino acid transport by shifting small amounts of K^+ into muscle cells, then it should be possible to inhibit the effect of growth hormone on amino acid transport by placing the muscle in a system which does not permit movement of K^+ into the cells. An experiment was performed to test this possibility. Hypophysectomized rat diaphragms were depleted of K^+ and then transferred to flasks containing K^+-free bicarbonate buffer, 0.01 M glucose, AIB-1-C^{14} and in some cases, bovine growth hormone (25 μg/ml) and incubated for 1 hour. K^+ measurements made on muscles similarly treated indicated that K^+ content of the diaphragmatic muscle remained constant during the AIB uptake period. Diaphragms which were incubated with growth hormone had a mean AIB distribution ratio of 2.97 ± .18 ($n = 4$), while the controls had a mean ratio of 1.03 ± .03 ($n = 3$), ($P < 0.01$). Thus diaphragms which were K^+ depleted and could not take up K^+ during the AIB uptake period still responded to bovine growth hormone with an acceleration of amino acid transport.

It is of considerable interest that the rib cage preparation of rat diaphragm does not lose K^+ during incubation *in vitro*. In one experiment normal rat diaphragms were incubated for 5 hours and still no change in K^+ was noted. Since total tissue water remained stable during incubation, the constant K^+ to wet wgt. ratio observed cannot be due to concomittant loss of K^+ and water. K^+ concentration of the medium increased by about 1 meq/l during the 2 hour incubation in 30 ml of Krebs buffer presumably due to a loss of K^+ from extraneous cut muscle tissue on the preparation. This increase in medium K^+ concentration was not responsible for the stability of the K^+ content of the diaphragm since lowering initial medium K^+ concentration from the usual 5.9 meq/l to 4.8 meq/l failed to cause any change in K^+ content after 2 hours' incubation. Previous studies which indicated that the rat diaphragm loses K^+ during incubation (*ibid.*) were made using the "cut" diaphragm preparation which is prepared by dissecting the diaphragmatic muscle away from the rib cage and tendon before incubation. It was found in the present study that when just a few muscle fibers are cut by bisecting the rib cage preparation anterioposteriorly, the diaphragm lost K^+ during incubation. Hence, it appears that the integrity of the muscle fibers is essential for maintenance of constant K^+ levels during incubation *in vitro*.

Summary. Experiments were performed to determine if hypophysectomy and growth hormone influence amino acid transport by rat diaphragm through alterations in muscle K^+ content. Hypophysectomy did not alter *in vivo* K^+ content of rat diaphragm. Neither hypophysectomy nor incubation with growth

hormone exerted a detectable influence on K^+ level of the diaphragm *in vitro*. Moreover, diaphragms which were K^+ depleted and could not take up K^+ during the incubation period, still responded to bovine growth hormone with an increase in amino acid transport. Thus it appears unlikely that the effects of hypophysectomy and growth hormone on amino acid transport by rat diaphragm are mediated through changes in muscle K^+ content.

The authors wish to thank Dr. Frank L. Engel for his interest and critical evaluation of the manuscript.

1. Kostyo, J. L., Hotchkiss, J., Knobil, E., *Science*, 1959, v130, 1653.

2. Kostyo, J. L., Engel, F. L., *Endocrinol.*, 1960, v67, 708.

3. Batts, A. A., Bennett, L. L., Garcia, J., Stein, J., *ibid.*, 1954, v55, 456.

4. Christensen, H. N., Riggs, T. R., *J. Biol. Chem.*, 1952, v194, 57.

5. Riggs, T. R., Walker, L. M., Christensen, H. N., *ibid.*, 1958, v233, 1479.

6. Oxender, D. L., Christensen, H. N., *ibid.*, 1959, v234, 2321.

7. Kipnis, D. M., Cori, C. F., *ibid.*, 1957, v224, 681.

8. Rixon, R. H., Stevenson, J. A. F., *Quart. J. Exp. Physiol.*, 1957, v42, 346.

9. Rixon, R. H., *Am. J. Physiol.*, 1958, v194, 363.

10. Randle, P. J., Smith, G. H., *Biochem. J.*, 1958, v70, 490.

Received April 10, 1961. P.S.E.B.M., 1961, v107.

The Effect of Metrazol in Isolated Mammalian Cells.* (26623)

GEORGE J. ALEXANDER AND RITA B. ALEXANDER (Introduced by Warren M. Sperry)
Dept. of Biochemistry, N. Y. Psychiatric Institute and College of Physicians and Surgeons, Columbia University, New York, N. Y.

In vitro studies in rat brain slices show that addition of Metrazol (pentamethylenetetrazole), inhibits 20-25% of the oxygen uptake(1). Studies of the mode of action of Metrazol in intact animals indicate that it stimulates oxygen consumption and causes symptoms akin to those of hypoxia(2). We decided to investigate the effect of Metrazol on a homogeneous cell population, propagated *in vitro*. Strain L mouse fibroblasts (3) were selected for the initial investigation because they are stable, sturdy, and can easily be grown in quantity.

Materials and methods. L strain, NCTC 929, mouse fibroblasts and all media were obtained from Microbiological Associates, Inc., Bethesda, Md. Metrazol was purchased from the Bilhuber-Knoll Corporation, Orange, N. J. Cells were maintained and incubated in Eagle's minimum essential medium (4) supplemented with 10% horse serum and a penicillin-streptomycin mixture. They were grown in monolayer cultures with three changes of medium a week. Harvested cells were introduced into a U-shaped chamber and grown in submerged aerated cultures with continuous inflow of fresh medium and outflow of spent broth. Agitation was sufficient to keep the cells dispersed and to prevent adhesion to the walls. Portions of the suspension were withdrawn at regular time intervals and viable cells were counted in a haemocytometer. Other portions of the suspension were transferred to Warbug vessels equipped with CO_2 traps for measurement of oxygen uptake.

Results. The normal oxygen uptake of strain L cells measured on portions of a suspension culture shaken in Warbug vessels is 0.25 μl per mg of cells per minute. Addition of Metrazol to the Warbug vessels brings about an immediate decrease in the oxygen uptake. Thirty minutes after addition of Metrazol (4×10^{-4} M) the rate of oxygen uptake decreases 94% (Fig. 1) and then remains constant for several hours. Comparison of the rates of oxygen consumption 30 min. after addition of various amounts of

* Supported in part by a grant from Nat. Inst. of Neurological Diseases and Blindness, N.I.H., Bethesda, Md.

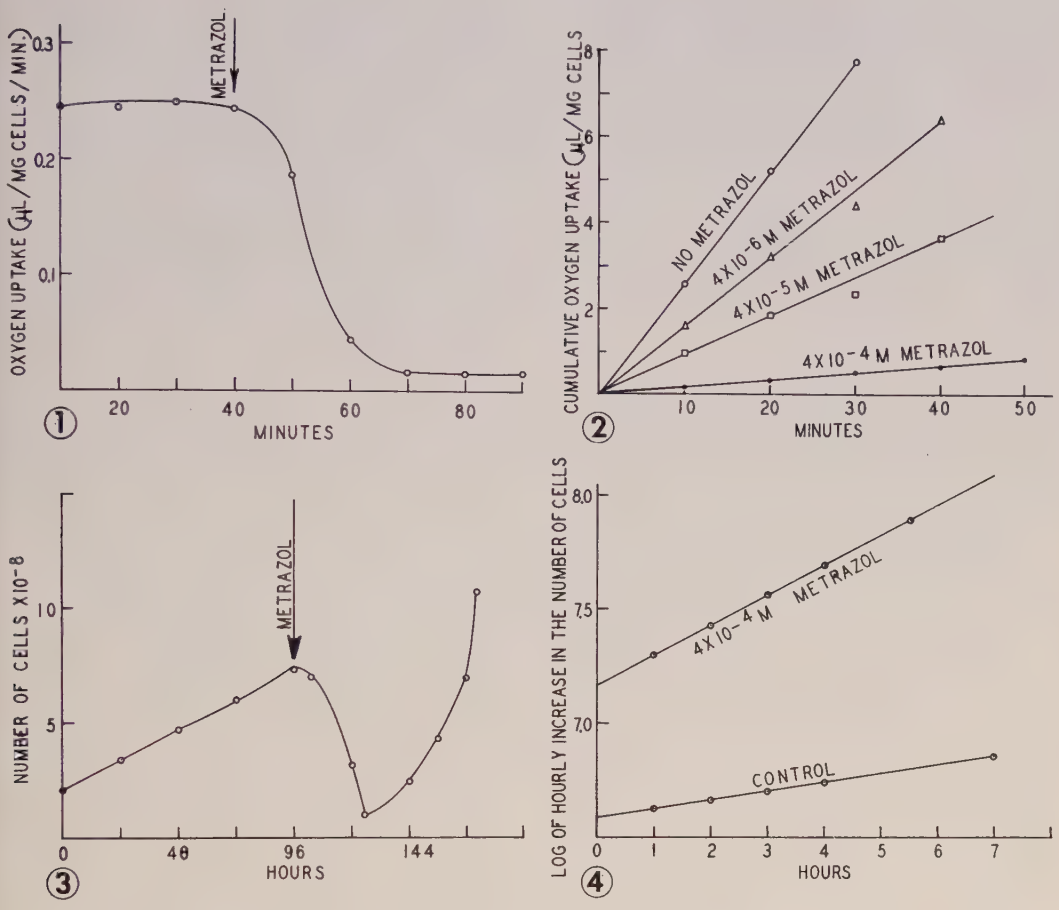


FIG. 1. Typical experiment showing decrease in Qo_2 after addition of Metrazol (4×10^{-4} M) to cell suspension in Warburg vessel.

FIG. 2. Oxygen uptake in presence of varying concentrations of Metrazol. Measurements began 30 min. after addition of drug to suspensions of cells in Warburg vessels.

FIG. 3. Initial decrease and eventual increase in number of cells in perfused culture after introduction of 4×10^{-4} M Metrazol.

FIG. 4. Growth rate of perfused culture 72 hours after addition of Metrazol.

Metrazol fails to reveal a threshold concentration below which there is no inhibition of oxygen uptake. Low concentrations of Metrazol continue to inhibit although the inhibition is reduced (Fig. 2).

Inhibition of oxygen consumption proves to be reversible, however, in perfused cultures. At first, addition of Metrazol at the level of 4×10^{-4} M, to a perfusion culture causes a sharp drop in the number of cells. This is followed by a reversal after 30 hours (Fig. 3). After the reversal, the rate of growth increases rapidly. As the log phase of growth is reached the generation time decreases to an unprecedented 7.5 hours (Fig. 4, Table I). The average generation time under similar conditions but in the absence of Metrazol is 13 times longer. Oxygen utilization increases also but only three-fold. The net effect of these changes is to reduce the

TABLE I. Metabolism of Perfused Culture Maintained for a Week in Presence of Metrazol.

	Control	Metrazol
Qo_2 (μl/mg cells/hr)	15.5 ± 0.6	46.1 ± 1.9
Generation time	94.6 ± 0.4	7.5 ± 0.8

Qo_2 measured in Warburg vessels on aliquots withdrawn from perfusion chamber. Generation time (No. of hours required for doubling cell population) calculated from counts of viable cells at hourly intervals.

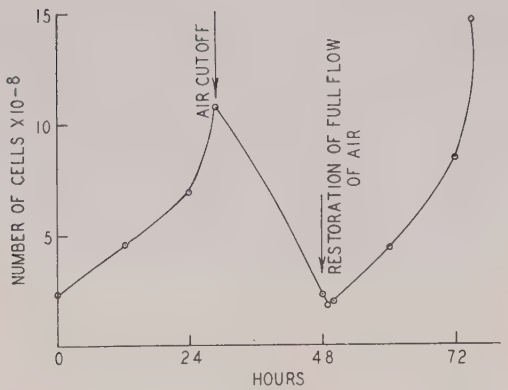


FIG. 5. Decrease in number of cells caused by 20% decrease in air flow through "Metrazol" culture and rebound effect after restoration of normal air flow.

consumption of oxygen per cell. This renders the culture extremely sensitive to small variations in oxygen supply. Decreasing the flow of air through the culture causes an immediate drop in the number of cells. Restoration of the full flow of air restores the rapid proliferation of cells (Fig. 5).

Discussion. Short-term tissue culture experiments indicate that Metrazol inhibits cell growth and thereby reduces oxygen uptake. This is in agreement with the findings of Kajdi and Williams in brain slices(1). However, in experiments of longer duration, which are possible with cell cultures but not with tissue slices, Metrazol stimulates rapid oxygen uptake and a more rapid growth of those cells which survive the initial inhibition. Since oxygen uptake does not keep pace with cell growth, the oxygen tension within the cells must be lower in cells growing in the presence of Metrazol than in control cultures. Possibly, the cell metabolism changes under the influence of Metrazol along the lines suggested by Warburg, who believes that when the respiration of a cell is gradually inhibited, the surviving cell compensates for the loss of energy by increasing the rate of fermentation(5). Lactic acid should accumulate in such a case, and indeed, Gourdjan *et al.* reported an accumulation of lactic acid in the brain during Metrazol-induced seizures(2). In a series of experiments with *S. cerevisiae,* which are capable of growing under either aerobic or anaerobic conditions, we found that the metabolic pattern of aerated yeast grown in the presence of Metrazol resembles the pattern of control cultures grown in the absence of oxygen (unpublished observations).

It is unlikely that adaptation of the cells to Metrazol involves synthesis of a detoxifying system capable of disposing of Metrazol. Since fresh Metrazol is continuously fed into the culture chamber, its removal would require too large an expenditure of energy. Synthesis of an active barrier preventing the penetration of Metrazol into the cytoplasm would also require a large expenditure of energy. On the other hand, it is possible that no adaptation but selection took place in our cultures and that cells which were originally resistant to Metrazol were the only ones to survive. In either case, whether through adaptation or selection, the average surviving cell differs considerably from the average prototype; it multiplies faster than the control cell but utilizes less oxygen during growth.

Summary. In short-term experiments, oxygen uptake by mouse fibroblast cells is inhibited by the addition of the convulsant Metrazol. After 30 hours of incubation in suspension culture, the effect of Metrazol is reversed. Both oxygen uptake and cell growth are enhanced. Cells grown in the presence of the drug multiply 13 times faster and use up 3 times as much oxygen as control cells.

The authors wish to express their gratitude to Dr. Warren M. Sperry without whose encouragement and support this work would not have been possible.

1. Kajdi, L., Williams, C. H., *J. Am. Pharm. Assoc.,* 1953, v42, 618.

2. Gourdjan, E. S., Webster, M. D., Stone, W. E., *A. Res. Nerv. and Ment. Dis. Proc.,* 1946, v26, 184.

3. Sanford, K. K., Earle, W. R., Likely, G. D., *J. Nat. Cancer Inst.,* 1948, v9, 229.

4. Eagle, H., *Science,* 1959, v130, 432.

5. Warburg, O., *Science,* 1956, v123, 309; v124, 269.

Received March 20, 1961. P.S.E.B.M., 1961, v107.

Effect of Prolonged Exposure to 15% CO_2 on Calcium and Phosphorus Metabolism.* (26624)

K. E. Schaefer, Mary Hasson and H. Niemoeller†
U.S.N. Medical Research Laboratory, New London, Conn. and Department of Pathology, Yale University School of Medicine, New Haven, Conn.

Brown and Prasad(1) reported slight increases in plasma calcium and inorganic phosphorus, associated with a fall in ultrafiltrable Ca, during acute exposure of dogs to high CO_2 concentrations (30-40% CO_2). Nichols, Schaefer and Carey(2) found in men during a chronic slight respiratory acidosis (exposure to 1.5% CO_2 for 42 days) a decrease in plasma calcium and increase in inorganic phosphorus during the period of uncompensated respiratory acidosis. In experiments reported here, calcium and phosphorus metabolism was studied during a period of chronic respiratory acidosis induced by exposure to 15% CO_2 in guinea pigs.

Methods. Male guinea pigs of the Hartley strain weighing between 400 and 550 g were exposed to 15% CO_2 in 21% O_2 for periods up to 73 days. Two groups of animals were studied after recovery on air for 1 and 11 days respectively following exposure to 15% CO_2 for 7 days. In a second series of experiments urine was collected daily in metabolism cages prior, during, and after exposure to CO_2, from 4 guinea pigs. The number of animals used in each experimental group is listed in Table I. Blood samples were taken under anaerobic conditions through heart punctures after the animal was stunned by a blow at the base of the skull. pH was measured in a constant temperature waterbath at 37°C by a model G Beckman pH meter using hypodermic glass electrodes. Whole blood CO_2 content was determined with the Van Slyke apparatus. Experimental chambers allowed good control of temperature and humidity (78°F ± 2° and 65-75% humidity). Hematocrit was measured using the microcapillary method. Blood plasma calcium and urinary calcium were determined by the method of Clarke and Collip, a modification of the procedure of Kramer and Tisdall(3). Blood calcium data after one hour exposure were obtained using the EDTA method of Munson *et al.*(4). The inorganic phosphorus in blood and urine was measured by the method of Fiske and Subbarrow as modified by Roe and Whitmore(5). Plasma protein was measured using the method of Wolfson, Cohn, Calvary and Schiba(6). The albumin/globulin ratio was determined in a limited number of blood samples in animals exposed to CO_2 for various time periods. Data on ultrafiltrable calcium were obtained by the method of Toribara *et al.*(7).

Results. Table I presents results on plasma pH, calcium, inorganic phosphorus, ultrafiltrable calcium and plasma protein. The pH shows a marked drop after one hour and one day of exposure to 15% CO_2, and increases to a level somewhat below normal but not significantly different after the second to third day. This indicates that after 2-3 days a compensation of the respiratory acidosis is reached. This is supported by other data on urine pH, plasma and urine electrolytes reported elsewhere(8). Plasma calcium rises slightly and inorganic phosphorus shows a small decrease after one day of exposure to 15% CO_2. On the second to third day and thereafter plasma calcium increases and plasma phosphorus decreases significantly. After 4-7 days and after 20 days of exposure calcium level is still elevated and phosphorus level remains lowered. Recovery on air for one day after exposure to 15% CO_2 for 7 days does not change this status. After 11 days of recovery, inorganic phosphorus has returned to initial levels while plasma calcium is somewhat lower than control values.

Ultrafiltrable calcium increases slightly during the first phase of CO_2 exposure and is significantly higher after 4-7 days of exposure and after one day recovery following

* This investigation was supported in part by O.N.R.

† With the technical assistance of J. R. Cassidy.

TABLE I. Effect of Prolonged Exposure to 15% CO_2 on Blood pH, Plasma Calcium, Inorganic Phosphorus, Ultrafiltrable Calcium and Total Protein.

		pH of blood	Plasma calcium, mg %	Plasma inorg. phosphorus, mg %	Total protein, g %	Ultrafiltrable calcium mMol/l	Ultrafiltrable calcium Fraction of total calcium
Control	Mean	7.38	11.6	7.0	8.4	1.31	.45
	S.D.	.05	.6	1.1	.14	.22	.07
	No.	25	15	20	8	8	8
15% CO_2 1 hr	Mean	7.07†	11.6	6.7	—	1.41	.54
	S.D.	.02	.6	.9	—	.10	.03
	No.	12	6	13	—	6	6
15% CO_2 1 day	Mean	7.21†	12.0	6.8	8.7	1.40	.48
	S.D.	.08	.9	.9	.5	.30	.09
	No.	38	14	11	12	9	9
15% CO_2 2-3 days	Mean	7.29†	12.9*	5.8†	8.5	—	—
	S.D.	.03	.6	.9	.9	—	—
	No.	25	13	17	8	—	—
15% CO_2 4-7 days	Mean	7.35	12.1	5.6†	8.4	1.69†	.60*
	S.D.	.06	.6	.7	.3	.20	.05
	No.	34	14	8	13	10	10
15% CO_2 20-73 days	Mean	7.34	13.2*	6.1†	8.6	—	—
	S.D.	.08	.6	.2	.25	—	—
	No.	12	13	10	4	—	—
7 day exp.							
15% CO_2 1 hr rec.	Mean	—	11.6	—	—	1.58†	.65*
	S.D.	—	.5	—	—	.14	.09
	No.	—	7	—	—	4	4
15% CO_2 1 day rec.	Mean	7.34*	12.4*	5.5†	—	1.64†	.50
	S.D.	.08	.4	.9	—	.24	.06
	No.	21	8	11	—	4	4
15% CO_2 11 day rec.	Mean	7.37	10.9	6.9	8.0*	—	—
	S.D.	.06	.5	1.0	.4	—	—
	No.	23	6	8	5	—	—

* Statistically significant from control values at the 5% level.
† " " " " " " " 1% " and greater.

7 days exposure. No changes in total protein were observed.

Fig. 1 shows measurements of daily urine volume, excretion of urinary calcium, and inorganic phosphorus obtained from a group of 4 guinea pigs prior to and during exposure to 15% CO_2, and during a recovery period on air following exposure. Urinary volume increases slightly during the first day of CO_2 exposure and decreases during the following days of exposure. After 5-6 days of recovery, control values are reached. A rise above initial levels is noted during the period of 6-11 days recovery. During the first phase of uncompensated respiratory acidosis, calcium as well as phosphorus excretion is significantly increased. Calcium excretion falls during the second phase of CO_2 exposure below control values, while phosphorus excretion shows a transitory decrease during the second and third day to initial levels followed

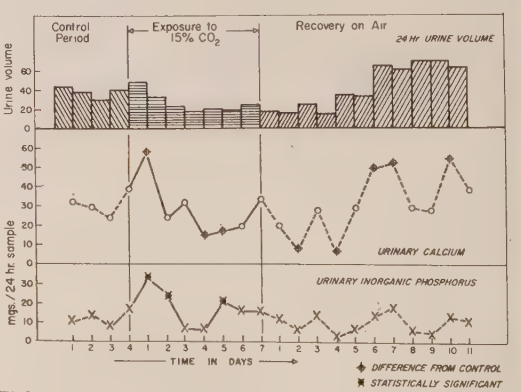


FIG. 1. Effect of prolonged exposure of guinea pigs to 15% CO_2 on 24 hour urine volume and urinary excretion of calcium and inorganic phosphorus.

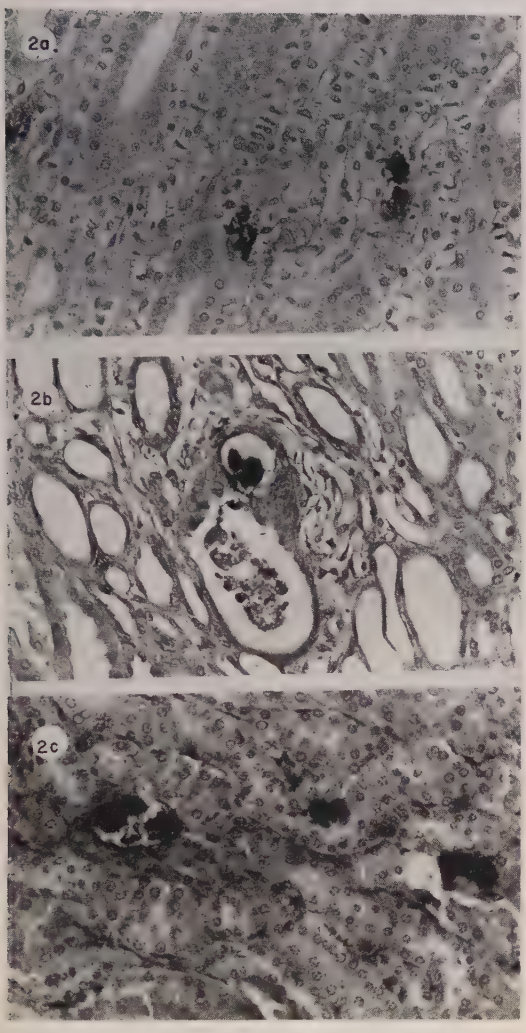


FIG. 2a. Interstitional calcium deposits in the renal medulla of a guinea pig exposed to 15% CO_2 for 7 days. 2b. Intra tubular calcium deposit in the renal cortex of a guinea pig exposed to 15% CO_2 for 73 days. 2c. Calcium deposits in tubular basement membranes of the renal cortex of a guinea pig exposed to 15% CO_2 for 47 days.

by a second significant rise during the fifth day of the exposure period when a value of 20.5 ± 7.2 was reached. Circled values indicate daily phosphorus and calcium excretion levels which differ from control values at a 5% level of confidence and greater.

Histopathologic findings. In connection with the evidence for changes in calcium and phosphorus metabolism occurring under chronic exposure to 15% CO_2 mention should be made of the finding of renal calcinosis in these animals. Our series of 22 test animals and 4 controls showed a fairly irregular occurrence of renal calcifications, starting as early as 48 hours after beginning of exposure. The lesions were never severe, except in 2 animals which were exposed for more than one month. The localization and type of the lesions appeared not to follow rigid pattern. Three main types of calcification were noted: 1) interstitial deposits, usually confined to the medulla and often in immediate vicinity of the tip of the papilla (Fig. 2a); 2) intratubular deposits, commonly in the cortex or medulla close to the corticomedullary junction. These were associated with changes in tubular epithelium (flattening, pyknosis of nuclei and basophilia of cytoplasm regeneration) as shown in Fig. 2b; 3) occasionally calcification originated in the tubular basement membranes, as shown in Fig. 2c, where the epithelium is lifted off its base and pushed into the lumen of the tubule. This lesion was located near the corticomedullary junction.

Discussion. The fall in plasma inorganic phosphorus associated with a rise in plasma calcium found during chronic respiratory acidosis in guinea pigs represents a major difference between the reported increase in inorganic phosphorus in response to acute exposure to CO_2 for periods of 2-4 hours(1). The changes in plasma calcium and phosphorus observed in our experiments are similar to those produced by parathyroid stimulation or injection of parathyroid extract(9). The increased urinary phosphorus excretion during the first and fifth day of exposure to 15% CO_2 indicates 2 phases of increased parathyroid activity. The second phase, however, could not be detected in plasma calcium and phosphorus values since these data were not collected on every single day of exposure. Reported findings agree with results of a study by Stanmeyer, King, Scofield and Colby(10) on rats. They observed, on the basis of daily measurements during a 7 day period of exposure to 15% CO_2, two phases at the end of the second and seventh day in which plasma calcium increased and plasma phosphorus decreased. Data obtained in 2 species of animals suggest development

of an increased parathyroid activity during chronic exposure to CO_2 which apparently does not occur in acute CO_2 exposure.

A decrease in pH, as found in respiratory acidosis, is known to produce a decrease in calcium binding of proteins(11) and a rise in ionized and ultrafiltrable calcium(12,13). The average value of ultrafiltrable calcium (controls) obtained in our experiments, 1.31 mM/l serum, agrees with data reported in the literature on mean ultrafiltrable calcium in normal animals amounting to 1.31 mMol/l(13). However, the mean percentage of total calcium observed, 45%, is smaller than the 53.3% given by Prasad (13). Ultrafiltrable calcium increased only slightly during the period of CO_2 exposure in which decrease in pH was greatest, one hour and one day exposure, and more significantly after 7 days' exposure and after one hour and one day recovery on air following 7 days' exposure. In the latter conditions pH changes were rather small. The other factors known to influence calcium protein interactions, albumin/globulin ratio and total protein, were not significantly altered. The inorganic phosphorus, however, was much lower. These data seem to support the hypothesis of Brown and Prasad(1) that an increase in inorganic phosphate produces a decrease in ultrafiltrable calcium and *vice versa*, probably due to formation of some nonfiltrable calcium phosphate compound, which is also in agreement with results of other investigations(14,15). However, definite proof of this theory would require measurements of ultrafiltrable inorganic phosphate and protein bound phosphate in the plasma *in vivo* under these experimental conditions. Although no fall in ultrafiltrable calcium was observed prior to the apparent parathyroid stimulation in the periods investigated, our data are not quite adequate to draw a conclusion as to whether CO_2 could be considered a stimulus to which the parathyroid gland responds directly.

Renal calcification in animals exposed intermittently and continuously for prolonged periods to increased concentrations of CO_2 have been reported by Meesen(16) and Zinck (17). Anoxemia was excluded as being a factor in development of these lesions. Zinck (17) noted that the correlation between concentration of CO_2, length of exposure, and severity of the renal lesion was poor, and that there was a great deal of individual variation. In extensive studies on experimental nephrocalcinosis, Goebel and Koburg(18) were able to produce renal calcium deposits by subjecting animals to respiratory insufficiency. The most frequent of the 3 types of renal calcification found in our experiments were the interstitial calcium deposits located in the inner medulla. A similar renal calcification located primarily interstitially in the medulla was reported in a case of parathyroid adenoma(19) while in another recent study(20) dealing with nephrocalcinosis in experimental hyperparathyroidism, interstitial calcification was described as "minimal".

The physiological data obtained in animals exposed to 15% CO_2 suggest that parathyroid stimulation plays a role in development of the reported renal lesions in chronic respiratory acidosis.

Summary. (1) During chronic respiratory acidosis, produced by prolonged exposure of guinea pigs to 15% CO_2, plasma calcium was found to increase and plasma phosphorus to decrease significantly. (2) These changes appear to be related to an increased parathyroid activity as evidenced by an increased urinary phosphorus excretion. (3) Ultrafiltrable calcium increased only slightly during the period of uncompensated respiratory acidosis with the greatest pH changes, and rose much higher during the period of compensated respiratory acidosis where pH changes were smaller. During the latter period plasma inorganic phosphorus values were much lower, supporting the hypothesis of Brown(1) concerning the inverse relationship of ultrafiltrable calcium and inorganic phosphorus. (4) Histological studies showed a significant renal calcification in guinea pigs exposed for prolonged periods to 15% CO_2.

1. Brown, E. B., Jr., Prasad, A. S., *Am. J. Physiol.*, 1957, v190, 462.

2. Nichols, G., Schaefer, K. E., Carey, Ch., NMRL Rep. 292, 1954.

3. Clark and Collip's Modification of the Kramer-Tidsall Method. Hawk and Bergeim, 1942, *Pract. Physiol. Chem.,* 11th Edition, 468.

4. Munson, E. L., Tseri, O. A., Kennedy, A. D., Cohn, V., Skips, M., *J. Dental Res.,* 1955, v34, 714.

5. Roe, J. H., Whitmore, E. R., *Am. J. Clin. Path.,* 1938, v8, 233.

6. Wolfson, W. A., Cohn, C., Calvary, E., Ichiba, F., *ibid.,* 1948, v18, 723.

7. Toribara, T., Terepla, A. R., Dewey, P. A., *J. Clin. Invest.,* 1957, v36, 738.

8. Schaefer, K. E., *Proc. Second Intern. Symposium on Submarine and Space Medicine,* Stockholm, 1960.

9. Albright, F., Ellsworth, R., *J. Clin. Invest.,* 1929, v7, 183.

10. Stanmeyer, W. R., King, C. T. G., Scofield, H., Colby, R., *Proc. First Intern. Symposium on Submarine and Space Medicine,* New London, Conn., 1958.

11. Carr, C. W., in Sheldowsky, T. (Ed.). *Electrochemistry in Biology and Medicine,* Chap. XIV, New York, John Wiley & Sons, Inc., 1955.

12. Peters, J. P., Van Slyke, D. D., *Quant. Clin. Chem., Methods,* vII.

13. Prasad, A. S., *AMA Arch. Intern. Med.,* 1960, v105, 560.

14. Hopkins, T., Howard, J. E., Eisenberg, H., *Bull. Johns Hopkins Hosp.,* 1952, v91, 1.

15. Terepka, A. R., Toribara, T. Y., Dewey, P. A., *J. Clin. Invest.,* 1958, v37, 87.

16. Meesen, H., *Arch. Path.,* 1948, v45, 36.

17. Zinck, K. H., *German Monograph on Submarine Medicine,* edited by K. E. Schaefer. Prepared by U. S. Naval Forces, Germany, Technical Section, Medical.

18. Goebel, A., Koburg, E., *Beitr. Path. Anat.,* 1959, v120, 111.

19. Anderson, W. A. D., Endocrinology, 1939, v24, 372.

20. Carone, F. A., Epstein, F. H., Beck, D., Levitin, H., *Am. J. Path.,* 1960, v36, 77.

Received January 3, 1961. P.S.E.B.M., 1961, v107.

Carbonic Anhydrase Activity in Fetal and Young Rhesus Monkeys.*† (26625)

DELBERT A. FISHER‡§ (Introduced by T. C. Panos)

Department of Pediatrics, University of Oregon Medical School, Portland

The distribution and physiologic significance of the enzyme carbonic anhydrase has been extensively investigated since the initial report of Meldrum and Roughton(1,2). In the mammalian species high activities have been measured in erythrocytes, renal cortex, gastric mucosa and pancreas(2,3); the enzyme appears to play an essential physiologic role in these tissues(2-5). Significant activities of unknown significance have been reported in brain, spinal cord, testis, salivary glands and the lens and retina of the eye (2,3).

Several reports have dealt with activity of carbonic anhydrase in human fetal tissues obtained by induced abortion and in premature and newborn infants(6-11). Enzyme activity in erythrocytes and/or kidney has been compared in fetal and premature subjects, full term infants, children and adults in several of these reports(7,9-11). The present investigation reports maturation of carbonic anhydrase activity in several tissues of the rhesus monkey from 75 days of gestation to one year of age.

Experimental. Thirteen fetuses of rhesus monkeys were obtained by cesarean section at known gestational ages of 75, 90, 120 or 150 days calculated from the known date of conception (conceptional age). (Average duration of gestation in the rhesus monkey is 165 days). Portions of pancreas, kidney cortex, lung, gastric mucosa and erythrocytes were weighed and immediately frozen. Lung and kidney tissues were perfused with 0.9% NaCl; sections of fetal gastric fundus were

* Supported by grants from Oregon Heart Assn., U.S.P.H.S. and Ross Laboratories.

† Presented in part before Western Society for Pediatric Research meeting, Santa Monica, Calif., Nov. 2 and 3, 1959.

‡ Irwin Memorial Fellow of Oregon Heart Assn.

§ Present address: Dept of Pediatrics, University of Arkansas School of Med., Little Rock.

weighed intact because separation of mucosa was impracticable. Heparinized cord blood was centrifuged in capillary tubes; erythrocytes were transferred to tared parafilm cups, weighed and immediately frozen.

Similar tissues were obtained at autopsy from 10 healthy 350-day-old rhesus monkeys. In these animals lung and kidney tissues were perfused with 0.9% NaCl as in the fetuses. Gastric mucosa and submucosa were dissected from the muscular components. Brain tissue was also obtained from these animals (predominantly gray matter) from the tip of the right temporal lobe. Erythrocytes were prepared as described for fetuses. Blood from 10 adult monkeys was obtained by venipuncture in heparinized syringes. Subsequently erythrocytes were prepared by centrifugation in capillary tubes and were weighed and frozen as described.

Carbonic anhydrase activity in frozen tissues was found to be stable over at least a 4-month period.

Eighty to 300 mg of tissue was homogenized using a pyrex tube with motor driven Teflon pestle with appropriate amounts of distilled water to produce 5 to 25 mg of tissue in 0.5 cc of homogenate. Fifteen to 25 mg of tissue was assayed in fetuses. In older animals 10 to 15 mg of non-erythrocyte tissue and 5 to 15 mg of erythrocytes was assayed.

Homogenates were centrifuged 10 minutes at 1500 RPM in a refrigerated centrifuge and placed in a refrigerator for immediate assay. Specimens were placed in the Warburg flasks immediately before each determination.

To evaluate erythrocyte contamination of tissues, hemoglobin content of the earliest assayed fetal and postnatal homogenates was determined as follows: Homogenates were diluted 1 to 10 with distilled water and centrifuged. The hemoglobin content of the supernatant was determined at 415 mμ on a Beckman DU spectrophotometer by comparison with a linear standard prepared with known hemoglobin solutions. Approximately one-third of tissue homogenates were directly assayed for hemoglobin content. Contamination did not exceed 2.5% by weight in any case. Hemoglobin contamination in the amount of 0.30 mg per ml of homogenate was easily detected with the naked eye, and tissue homogenates were prepared to contain at least 20 mg non-erythrocyte tissue per ml so that 2% contamination was easily detectable. Thus in the remainder of specimens only homogenates with visible hemoglobin contamination were assayed directly. In no case did hemoglobin contamination exceed 2.5%.

Assays were carried out by a modification of the Krebs and Roughton Warburg technic (12,13). Twenty ml Warburg flasks with magnet attached 0.3 ml sidearms were used. The apparatus was equipped with a motor and eccentric wheel for shaking. One and eight-tenths (1.8) ml of phosphate buffer (3 parts of 0.1 M Na_2HPO_4 and 2 parts of 0.1 M KH_2PO_4) with 0.5 ml of plain distilled water (blanks) or distilled water homogenate was placed in the Warburg flask; 0.3 ml of 0.33 M $NaHCO_3$ was placed in the sidearm. Each specimen was run with a blank as control. pH of the phosphate buffer was 6.8 at 20°C.

CO_2 evolution was measured after 10 minute equilibration at 0.2 to 0.4°C (ice bath) and CO_2 evolution read at 30 second intervals for 5 minutes. The average per minute CO_2 evolution in microliters was determined as the average per minute rate of CO_2 evolution during the first 3 minutes multiplied by the flask constants in the usual manner. There was a linear relationship between the activity (rate of CO_2 evolution per minute) and the logarithm of enzyme concentration over the range of homogenate concentrations utilized. This linear relationship is demonstrated for a partially purified carbonic anhydrase preparation and for erythrocyte and gastric tissue homogenates in Fig. 1.‖ Results were expressed in Meldrum-Roughton units (MRU). One MRU is the amount of enzyme to double the rate of the uncatalyzed reaction. The uncatalyzed reaction achieved a mean rate of 20 μl CO_2 evolution per minute by 3 to 4 minutes of reaction time. Thus

‖ The partially purified enzyme was obtained from Mann Laboratories (Lot #A-6836) prepared by the method of Keilin and Mann(14).

TABLE I. Carbonic Anhydrase Activity in Monkey Tissues.*

	Age, days	No. animals	Tissue activities RBC	Kidney	Stomach	Lung	Pancreas	Brain
Fetuses†	75	2	.03 .02	.03 .03	.07 .04	.03 .01	.02 .00	
	90	2	.08 .07	.04 .06	.08 .06	.02 .02	.02 .00	
	105	4	.11 ±.03	.10 ±.05	.11 ±.02	.03 ±.01	.01‡	
	120	2	.06‡	.07 .15	.07 .06	.04 .02		
	150	3	.19 ±.09	.09 ±.03	.07 ±.03	.02‡		
Postnatal animals	350	10	.74 ±.34	.21 ±.02	.17 ±.04	.06 ±.02	.07 ±.03	.04 ±.02
	Adult	10	.72 ±.32					

* Enzyme activity recorded in Meldrum-Roughton units/mg wet tissue. Mean and stand. dev. recorded for 3 or more specimens.
† Age determined from known date of conception.
‡ Single specimens.

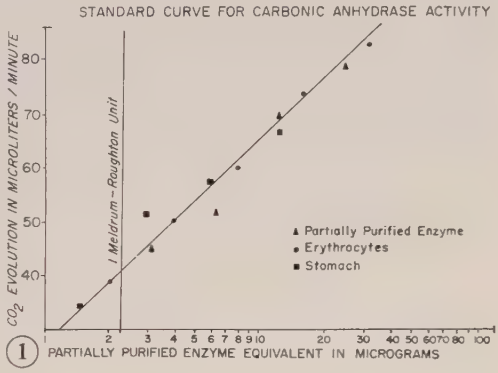
one MRU was equivalent to 2.3 μg of the partially purified enzyme preparation.

The MRU in the present system differs from the original descriptions which compared the catalyzed and uncatalyzed reaction rates during the second quarter of the reaction (E units). The MRU here employed compares the average per minute CO_2 evolution rate in the catalyzed reaction during the first 3 minutes with the stabilized uncatalyzed rate achieved by 3 to 4 minutes.

Reproducibility of the assay method as described is within ± 15%, which agrees with previously reported experience with the manometric technic(3,7).

Results. Table I presents the data derived from this investigation. The seeming rise in carbonic anhydrase activity of kidney and gastric tissues in the 105 day fetuses is of unknown significance and may relate to the small number of samples involved. There is a relatively greater carbonic anhydrase activity of erythrocytes, kidney and stomach, which progressively increased to age one year. This maturation of activity is shown graphically in Fig. 2.

As noted in Fig. 2 there is a 4-fold increase in erythrocyte (RBC) activity from 105 days of gestation to roughly one year of age in these animals and an increase in gastric and renal tissue activity of approximately 1½ times over the same period. Lung, pancreas and brain tissue activities remained low throughout these investigations. At 350 days



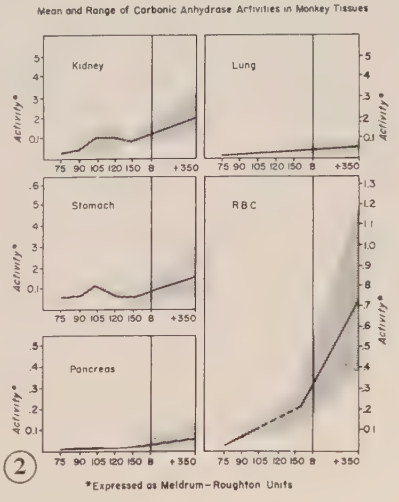


FIG. 1 and 2.

The erythrocyte and gastric curves in Fig. 1 are arbitrarily placed on the abscissa to demonstrate the similar linearity of the curves. Points on these curves represent serial dilutions of homogenate expressed as logarithm of tissue weight per 0.5 cc homogenate.

of age, activity of these tissues is equal to 1/10 to 1/20 of that found in erythrocytes. Adult human erythrocyte activity by the present method averages 15% more than activity in adult rhesus monkey erythrocytes.

Discussion. Erythrocyte carbonic anhydrase activities have been reported to be low in the human fetus and in premature infants. The activity increases 4- to 10-fold by adulthood(6,7,9,10). Berfenstam reported human erythrocyte activity to be about one-half of adult values by one year of age(9). Moreover, it has been observed that fetal tissue activities (kidney, gastric mucosa, pancreas, lung, liver and spleen) at various gestational ages were generally comparable to fetal erythrocyte activities and in many instances exceeded erythrocyte values(7,8).

The present results are in general agreement with these reported relative values. Erythrocyte carbonic anhydrase activity increased roughly 4-fold from 150 days of gestation to 350 days of age (equivalent to adult levels) and increased roughly 10-fold from 90 days of gestation to 350 days of age. Increases also occurred in renal and gastric tissue in contradistinction to the report of Day and Franklin in humans (also expressed as activity per mg wet weight of tissue)(7). Yaffee noted a 50% increase in carbonic anhydrase activity from newborn to adult human kidneys. These results were expressed on the basis of dry weight(11). Mean renal and gastric activity in the present study increased approximately 1½ times between 150 days of gestation and 1 year of age. Expressed on a dry weight basis values would be expected to increase only about 40% during this time in terms of the decrease in body water.¶ Thus the present results are in agreement with those of Yaffee in humans (11).

Direct comparison of reported values is difficult, however, because of dissimilar activity units, variation in reaction temperature and substrate concentration and variations in tissue preparation and homogenate concentrations. Adult human erythrocytes were observed in the present system to average 15% higher activity than adult monkey erythrocytes. Thus, human and rhesus monkey RBC activities appear similar, and the MRU in the present report is relatively greater than that in previous reports as summarized by Berfenstam(9). The present unit most closely approximates that of Meldrum and Roughton(1).

Summary. Maturation of carbonic anhydrase activity has been investigated in erythrocytes, gastric mucosa, kidney cortex, pancreas and lung tissues of the rhesus monkeys from 70 days of gestation to one year of age. Activity of brain tissue (cortical gray matter) was also assayed in the older animals. Significant maturation of activity in erythrocytes, gastric mucosa and renal cortex was noted during the investigation. Definite activity seemed present in fetal lung and pancreas, but values in the one year animals were minimal as were cerebral gray matter activities at one year. These results are in general agreement with reported studies in the human fetus and infant.

¶ Results were expressed on the basis of mg wet weight of tissue. Values for relative water content of monkey fetal tissues are not available but considerable information is available regarding water content of human fetuses, newborn infants and older children(15). Body water expressed as per cent body weight averages 89% at 4 months of gestation (roughly equivalent to 75 days of gestation in the monkey), 75% at birth and 65% at 2 to 3 years of age (roughly equivalent to 1 year in the monkey). Presuming similar relative content of body water in monkey tissues, the observed enzyme activities in tissue homogenates would have been expected to increase 2- to 2½-fold at birth and roughly 3-fold at 1 year of age over the values at 75 days of gestation if expressed on a mg dry weight basis. Water content of erythrocytes will vary minimally.

1. Meldrum, N. U., Roughton, F. J. W., *J. Physiol.*, London, 1933, v80, 113.
2. Davenport, H. W., *Physiol. Rev.*, 1946, v26, 560.
3. VanGoor, H., *Enzymologia,* 1948-49, v13, 73.
4. Davies, R. E., *Metabolic Aspects of Transport Across Cell Membranes,* Q. R. Murphy, ed., Univ. Wisconsin Press, Madison, 1957, p277.
5. Hollander, B., *Am. J. Physiol.*, 1953, v174, 191.
6. Stevenson, S. S., *J. Clin. Invest.*, 1943, v22, 403.
7. Day, R., Franklin, J., *Pediatrics,* 1951, v7, 182.
8. Berfenstam, R., *Acta Paediat.*, 1952, v41, 310.
9. ———, *ibid.*, 1952, v41, 32.

10. Altschule, M. D., Smith, C. A., *Pediatrics,* 1950, v6, 717.
11. Yaffee, S. J., *A.M.A. Am. J. Diseases Child.,* 1959, v98, 659.
12. Krebs, H. A., Roughton, F. J. W., *Biochem. J.,* 1948, v43, 550.
13. Waygood, E. R., *Methods in Enzymology,* S. P. Colowick and N. O. Kaplan, ed., Academic Press, New York, 1955, v2, 836.
14. Keilin, D., Mann, T., *Biochem. J.,* 1940, v34, 1163.
15. Friis-Hansen, B., Changes in Body Water Compartments During Growth, *Acta Paediat.,* 1956, v46, suppl. 110.

Received January 9, 1961. P.S.E.B.M., 1961, v107.

In vitro Metabolism of C-14 Labeled β-Carotene.* (26626)

R. F. KRAUSE
Department of Biochemistry, West Virginia University Medical Center, Morgantown

Previous studies in this laboratory(1) and others(2,3) on the *in vivo* metabolism of C-14 labeled β-carotene have indicated that radioactivity in the rat is found in the non-saponifiable fraction, fatty acid and water soluble fractions isolated from liver and extra-hepatic tissue. The expired CO_2 is also found to be radioactive.

It has been well demonstrated that β-carotene is converted to Vit. A by the intact animal but it is questionable if such a conversion can be demonstrated by isolated tissue preparation. In view of this possible difference between *in vivo* and *in vitro* metabolism of β-carotene it seemed worthwhile to investigate the *in vitro* metabolism of C-14 labeled β-carotene.

Materials and methods. The β-carotene† used in these experiments was produced by growing *Phycomyces blakesleeanus* in a suitable C-14 acetate medium(4). Carotene samples used were recrystallized from methanol and petroleum ether to a constant specific activity and they had an $E^{1\%}_{1\,cm}$ of 2535 at 450 mμ in light petroleum ether. Booth(5) suggests that 2550 is the representative value for pure β-carotene.

Carotene suspensions were prepared by using 40 mg of Tween 80 and an appropriate volume of buffer solution. The amount of carotene used in each incubation is given in the tables.

All animals were fasted 24 hours before use. Wistar strain, white female rats, weighing between 110-125 g, two rabbits weighing approximately 2 lb each, and four 2-week old chicks were used in these experiments. Animals were decapitated and allowed to bleed freely. The organs were quickly removed, blotted, and various types of homogenates prepared.

Whole homogenates were prepared by taking one-gram samples of organ and homogenizing it for 90 seconds in 2.5 ml of cold 0.25 M sucrose with a loose fitting glass Potter-Elvehjem homogenizer. Cell-free homogenates were prepared by centrifuging whole homogenates, as prepared above, at 800 × g at 0°C for 10 minutes and the supernatant fraction was used. Protein content of the latter fractions was determined by the method of Siekevitz(6).

Mitochondria samples equivalent to one gram of tissue were prepared according to the method of Schneider and Hogeboom(7) and suspended in 2.5 ml of 0.25 M sucrose.

Each standard incubation mixture contained 2 ml of a carotene suspension (see tables for amount of carotene used) in Tris buffer (veronal, Table I), 2 ml of tissue homogenate (representing 1 g of tissue or its equivalent) suspended in 0.25 M sucrose and 6 ml of 0.2 M Tris hydrochloride, pH 7.5 (veronal buffer pH 8.5 used for experiments in Table I). Two mg of penicillin G and

* Supported by a grant from Nat. Vitamin Foundation, New York.

† The labeled carotene was made available by a grant from Rockefeller Foundation to Dept. of Biochemistry and Plant Pathology, West Virginia Univ.

TABLE I. Destruction of C-14 Labeled Carotene and Incorporation of Radioactivity into Sterols, Saponifiable Material and Steam-Distillable Compounds by Whole Homogenates of Rat Organs.

The incubation media contained 800 μg (50,000 DPM) of C-14 labeled carotene, one g of whole organ homogenate in 10 ml of veronal buffer, pH 8.5 plus 0.05 M α-tocopherol and 0.1 M ascorbic acid. Flasks were shaken anaerobically in a water bath at 37° for 6 hr. All organs except adrenals came from the same rat. One g of the adrenal gland was obtained from pooled rats of similar strain, age and weight.

Organs used	Carotene recovered, μg	% destruction	Total N.S.*	DPS†	SF‡	SDF§	% activity recovered
			DPM				
Kidney	720	10	47,500	100	800	70	97
Skeletal muscle	680	15	47,000	110	1000	60	96
Pancreas	688	14	47,200	108	900	70	96
Adrenals	640	20	47,000	150	1500	80	97
Small intestine	656	18	46,800	112	1000	75	96
Spleen	672	16	47,100	107	1400	70	97
Liver	680	15	45,900	200	1500	85	95
No tissue	776	3	49,000	0	0	0	98

* Non-saponifiable material. † Digitonin-precipitable sterols. ‡ Saponifiable fraction. § Steam-distillable fractions.

1 mg of streptomycin phosphate were added to each homogenate, placed in a 50 ml Erlenmeyer flask and shaken in water bath at 37° for 6 hours. Determinations were made in duplicate.

The non-saponifiable fraction (NSF) was obtained by saponifying with 10 ml of 2 N alcoholic KOH for 2 hours, adjusting the alcohol concentration to 50%, and extracting with light petroleum ether. To insure complete separation of the non-saponifiable fractions the above procedure was repeated and appropriate extracts combined.

Digitonin-precipitable sterols (DPS) were isolated from the non-saponifiable fractions according to the procedures of Kelsey(8). Extinction values of the petroleum ether solution from the non-saponifiable fraction were measured at 450 mμ and amount of carotene calculated.

After the non-saponifiable material was extracted, the saponifiable fraction (SF) was prepared according to the procedure of Srere *et al.*(9). The extracted saponified product was repeatedly resaponified, acidified, extracted and washed to a constant specific activity. The saponifiable content was determined by weight.

The remaining acidified water soluble material was subjected to steam distillation and the steam-distillable fractions collected (SDF).

Aliquots of the above fractions were oxidized to CO_2, counted in a Model 6000 Dynacon Electrometer and reported in disintegrations per minute (DPM) per flask. Technical difficulties made it impractical to count the water soluble residue remaining after steam distillations.

Suitable controls were run with each set of experiments. One set of controls was run without tissue and another set used tissue previously heated to 100°C for 5 minutes. In these controls no significant radioactivity was detected in the digitonin-precipitable sterols, saponifiable material or steam-distillable fractions. Carotene recovery in the controls was approximately 98%.

The CO_2 liberated (Table III) was measured by connecting the incubation flasks directly to the gas chamber of the Dynacon. The entire system was under reduced atmospheric pressure throughout the incubation period. The pH of the mixture at end of incubation was 6 and addition of acid did not liberate additional labeled CO_2.

Results. The destruction of carotene by various whole tissue rat homogenates incubated for a 6-hour anaerobic period ranged between 10 and 20% (Table I). Preliminary studies showed that shorter incubation time resulted in less carotene destruction and that more carotene was destroyed when larger amounts of tissue were used. Adrenal and

TABLE II. Effect of Adding Various Cofactors to Cell-Free Rat Liver Homogenates on Destruction of Carotene.

Each incubation mixture contained 388 μg of carotene and a cell-free homogenate prepared from one g of rat liver (156 mg of protein). The medium was buffered with 0.2 M Tris hydrochloride to a pH 7.5 and made to a final vol of 10 ml. When cofactors were added their concentrations were: .05 M α-tocopherol; .1 M ascorbic acid; .0008 M DPN; .0015 M ATP; .0048 M $MgCl_2$ and .03 M nicotinamide. All incubation flasks were shaken aerobically in a water bath at 37° for 6 hr.

Incubation conditions	Carotene recovered, μg	% destroyed
Cell-free homogenate + Tris buffer	347	10.5
Idem + ascorbic acid	349	10.0
" + α-tocopherol	352	9.3
" + nicotinamide	347	10.5
" + ATP	340	12.4
" + DPN	342	11.9
" + $MgCl_2$	347	10.5
" + ATP + DPN + $MgCl_2$ + nicotinamide	328	15.5
" + α-tocopherol + ascorbic acid + ATP + DPN + $MgCl_2$ + nicotinamide	329	15.2

intestinal homogenates showed the greatest destructive activity. More activity was found in the saponifiable portion than in the digitonin-precipitable sterol or steam-distillable fractions.

To acquire optimum conditions for carotene destruction various cofactors were added to cell-free liver homogenates (Table II). The combined addition of ATP, DPN, nicotinamide and $MgCl_2$ produced maximum carotene destruction over a 6-hour period. The amount of carotene destroyed was approximately the same at a pH of 7.4 or 8.5.

The destruction of β-carotene and incorporation of radioactivity into the digitonin-precipitable sterols, saponified material and steam-distillable fractions by cell-free homogenates were not appreciably different for either aerobic or anaerobic conditions.

Equivalent amounts of whole liver homogenate, cell-free rat liver homogenate, and mitochondria appeared to be equally effective in destroying carotene and in their ability to incorporate radioactivity into the different isolated fractions.

Whole liver homogenates from several different species exhibited little difference in their ability to metabolize carotene.

The incubation of cell-free rat liver homogenate with labeled β-carotene produced some labeled CO_2 (Table III).

All measurements reported in tables are averages from duplicate incubations. The maximum variation between duplicate samples for carotene determination was 5% and 10% for all radioactive measurements in terms of DPM.

Discussion. The incubation of C-14 labeled β-carotene with various tissue preparations resulted in a negligible oxidation of carotene to CO_2 and incorporation of radioactivity into saponifiable material, digitonin-precipitable sterols, and steam-distillable compound(s). The saponifiable material from several incubation experiments was combined and the lead salts isolated according to the technic of Twitchell(10). The lead salts so isolated were found to have appreciable radioactivity.

It is recognized, in reporting the per cent carotene destroyed in the incubation experiments, that these values may not represent the amount of pure or unaltered carotene remaining after incubations. Since the amount of carotene present is determined spectrophotometrically at 450 mμ, it is possible that some early degradation product may also be affecting the light absorption at this wave length. The amount of yellowish non-saponifiable material present in rat liver did not appreciably affect the recovery of carotene

TABLE III. Liberation of CO_2 by Anaerobic Incubation of Cell-Free Rat Liver Homogenate.

The incubation mixture contained 500 μg (70,000 DPM) of C-14 labeled carotene, cell-free rat liver homogenate from one g of rat liver (170 mg protein), 0.2 M Tris hydrochloride buffer at pH 7.5 to a vol of 10 ml and the following added: .0048 M $MgCl_2$; .03 M nicotinamide; .0008 M DPN; and .0015 M ATP.

Incubation time, hr	CO_2 released DPM
1	30
2	180
3	60
4	40
5	20
6	0
Total period	330

as demonstrated by the approximate 98% recovery of added carotene to various controls.

The examination of radioactivity of the non-saponifiable fraction after 6 hours of incubating carotene with cell-free liver homogenate reveals considerable activity in a fraction which does not contain carotene or digitonin-precipitable sterols. It was not possible under these conditions to recover C-14 labeled Vit. A. Work is now in progress to isolate and characterize the various radioactive products present in this non-saponifiable fraction.

The liberation of $C^{14}O_2$ early in the incubation periods indicates that some cleavage of the carotene molecule takes place early in the degradation phase.

Addition of ATP, DPN, nicotinamide and $MgCl_2$ accelerated the destruction of carotene. Although there was considerable variation between one rat liver and another, the addition of these cofactors did accelerate the destruction of carotene by any one liver preparation. The amount of carotene destroyed by cell-free rat liver homogenates appeared to be approximately the same under aerobic and anaerobic conditions.

Summary. Ten to 20% of β-carotene was destroyed by incubating C-14 labeled β-carotene with various tissues from the rat. All tissues studied had the ability to transfer radioactivity from β-carotene to sterols, saponifiable material and steam-distillable compound or compounds. ATP, DPN, nicotinamide and $MgCl_2$ were cofactors that increased β-carotene destruction by cell-free homogenates. A negligible amount of CO_2 was liberated during incubation.

The author thanks Mrs. Phyllis Spiker for technical assistance.

1. Krause, R. F., Sanders, P. L., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 549.

2. Willmer, J. S., Laughlaud, D. H., *Can. J. Biochem. Physiol.,* 1957, v35, 819.

3. Fishwick, M. J., Glover, J., *Biochem. J.,* 1957, v66, 36.

4. Lilly, V. G., Barnett, H. L., Krause, R. F., Lotspeich, F. J., *Mycologia,* 1958, v50, 862.

5. Booth, V. H., *Carotene—Its Determination in Biological Material.* W. Heffer & Sons, Ltd., Cambridge, 1957.

6. Siekevitz, P., *J. Biol. Chem.,* 1952, v195, 549.

7. Schneider, W. C., Hogeboom, G. H., *Cancer Res.,* 1951, v11, 1.

8. Kelsey, F. E., *J. Biol. Chem.,* 1939, v127, 15.

9. Srere, P. A., Chaikoff, I. L., Daube, W. G., *ibid.,* 1948, v176, 829.

10. Twitchell, E., *Ind. Eng. Chem.,* 1921, v13, 806.

Received January 18, 1961. P.S.E.B.M., 1961, v107.

Action of Synthetic Lysine Polypeptides on Isolated Guinea Pig Ileum.* (26627)

T. RICHARDSON† AND M. A. STAHMANN
Department of Biochemistry, University of Wisconsin, Madison

Synthetic lysine polypeptides or polylysines are polycations which readily interact with many polyanionic substances. *In vitro,* they agglutinate bacteria(1,2), viruses and virus deoxyribosenucleic acid(3), and red blood cells(4,5). Lysine polypeptides inhibit pepsin(6) and fibrinolysis(7), but enhance conversion of fibrinogen to fibrin(8), and are hydrolyzed by trypsin(9). *In vivo,* polylysine preparations possess antiviral(10) and

* This report is based on a portion of a Ph.D. thesis by T. Richardson and was supported in part by research grant from Nat. Microbicl. Inst., N.I.H., U.S.P.H.S., and the Research Committee, Graduate School, Univ. of Wisconsin, from funds supplied by Wisconsin Alumni Research Foundation. Approved for publication by Director of Wisconsin Agr. Exp. Station.

† Predoctoral Research Fellow of Nat. Cancer Inst. Present address: Dept. of Food Science and Technology, Univ. of California, Davis.

antitumor(11) activities and are weakly antigenic in rabbits(12,13). Subcutaneous injections of polylysine into rats increases capillary permeability, presumably as a direct action on the vascular endothelial cement (14). A preliminary abstract(15) suggested that polylysine and protamine released histamine when large doses were injected intravenously into dogs or cats. Histamine was also released from washed cell-free liver homogenates. In the work reported here, we have continued studies on the biological properties of polylysine and will describe the release of a contractile substance, probably histamine, by lysine polypeptides from the isolated guinea pig ileum, preparation of polylysyl histamine, and the inability of trypsin to hydrolyze a terminal lysyl-histamine bond.

Materials and methods. Materials were obtained as follows: L-lysine from Mann Biochemicals, protamine sulfate from Eli Lilly Co., histamine from Distillation Products, and salt-free crystalline trypsin from Pentex Laboratories, Inc. Benadryl (2-diphenylmethoxy-N, N-dimethylethylamine hydrochloride) was purchased locally as a 1:100 solution for injection. Young adult female guinea pigs were obtained from Zeimet Farms, Madison.

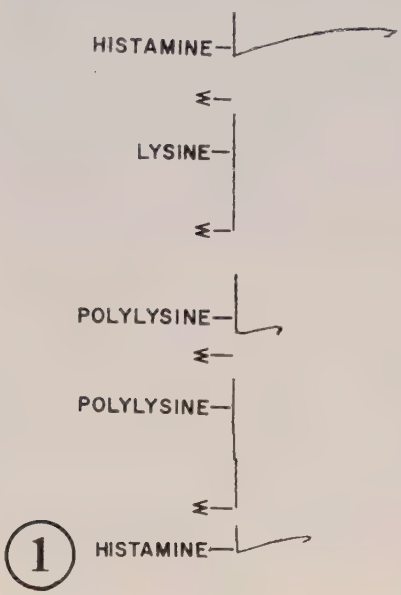
ϵ-Carbobenzoxy L-lysine polypeptide was prepared by ammonia-initiated polymerization of ϵ-carbobenzoxy-N-carboxy-L-lysine anhydride in dry dioxane(16). Degree of polymerization was determined by α-amino nitrogen analysis. Carbobenzoxy protective groups were removed by treatment with anhydrous hydrochloric acid in glacial acetic acid(17) and the product isolated as polylysine hydrochloride.

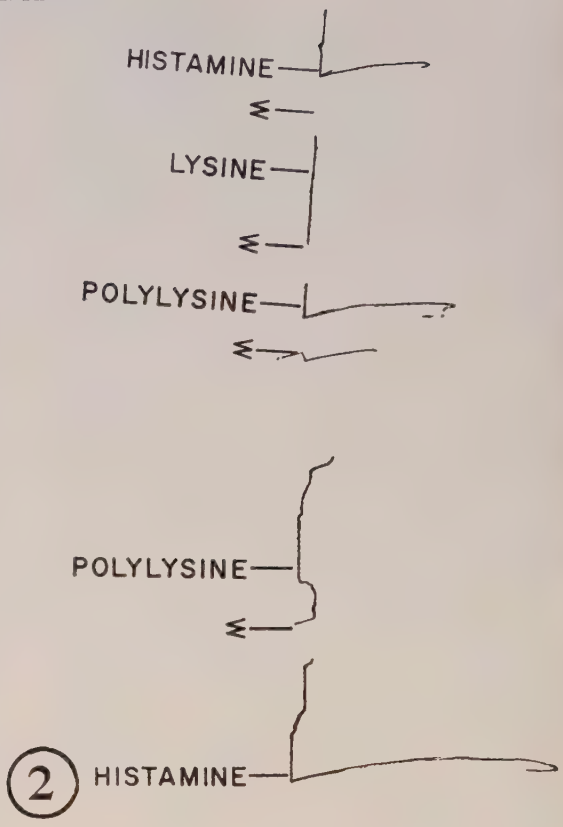
Poly-L-lysyl histamine was prepared by initiating polymerization of ϵ-carbobenzoxy-N-carboxy-L-lysine anhydride with histamine in dry dimethyl formamide. In the polymerization procedure, 15.3 g (50mM moles) of the anhydride were dissolved in 275 ml of anhydrous dimethyl formamide. To this solution were added 556 mg (5mM moles) of histamine base representing an anhydride to initiator ratio of 10. The clear yellowish solution was stirred for 36 hours, protected with a calcium chloride drying tube. Subsequently, the polymerization mixture was poured into excess water to precipitate the polymer. The product was washed thoroughly with water and lyophilized. Carbobenzoxy groups were removed with anhydrous hydrochloric acid(17) to yield poly-L-lysyl histamine hydrochloride in a yield of 84% of theory. The product gave strong positive Ehrlich diazo and biuret reactions.

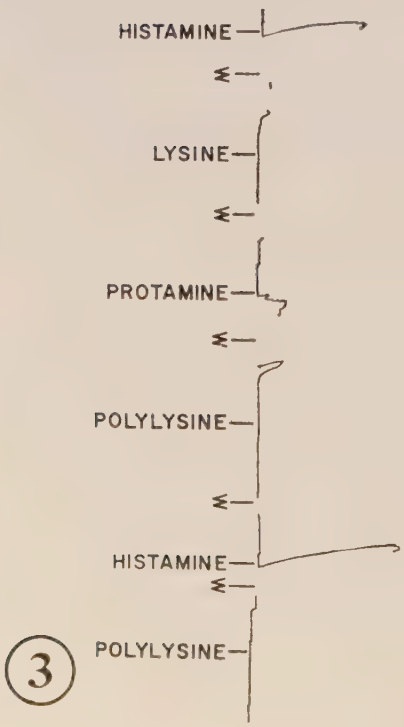
Polylysyl histamine, its hydrolysate, lysine, and histamine were spotted on Whatman 4 paper, and paper chromatograms were developed 16 hours in secondary butanol-98% formic acid-water (70:15:15). Papers were sprayed with 0.5% ninhydrin in water-saturated n-butanol or with the imidazole reagent of Block *et al.*(18). Polylysyl histamine did not migrate while histamine, lysine, and hydrolytic products migrated as expected. Furthermore, histamine when mixed with polylysyl histamine migrated freely. Although the solvent system did not effect good separation of L-lysine from histamine, it showed there was very little contamination of the polymer with lysine or histamine.

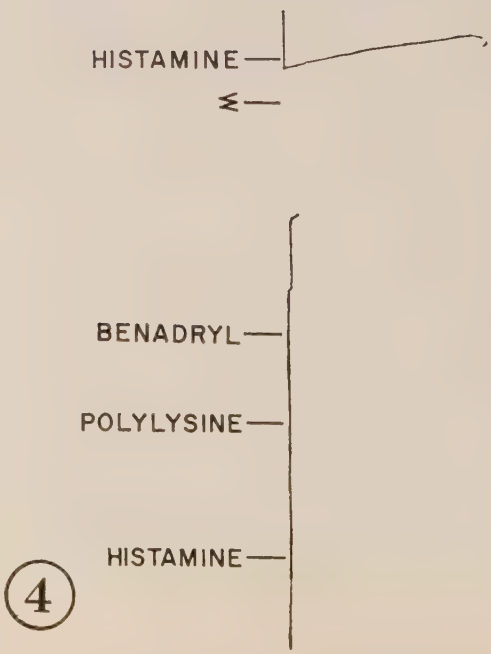
Polylysyl histamine was analyzed for histamine by adaptation of the colorimetric determination of histidine described by Jorpes (19). Diazotized sulfanilic acid and histamine formed a cherry-red color that reached maximum intensity in 10 minutes and had a maximum absorption at 500 mμ. Lysine did not interfere with the color measurement at this wavelength. Polylysyl histamine, hydrolyzed in 6N HCl, contained 4.5% histamine indicating an average of 15 lysyl residues per molecule.

The polypeptides were tested on the isolated guinea pig ileum using the technic as outlined by Code and McIntire(20). Sections of ileum 4 to 6 cm were mounted in Tyrode's solution gassed continuously with 95% oxygen and 5% carbon dioxide, U.S.P. The muscle bath, similar in design to Coulson's(21), had a 30 ml capacity and was immersed in a water bath at 35°C. Solutions to be assayed were incubated at 35°C, and test aliquots of never more than 1 ml were added to the muscle bath with a


HISTAMINE
LYSINE
POLYLYSINE
POLYLYSINE
1
HISTAMINE


HISTAMINE
LYSINE
POLYLYSINE
POLYLYSINE
2
HISTAMINE


HISTAMINE
LYSINE
PROTAMINE
POLYLYSINE
HISTAMINE
3
POLYLYSINE


HISTAMINE
BENADRYL
POLYLYSINE
4
HISTAMINE

syringe. After the muscle response was recorded on a Bird Horizontal Kymograph, the bath was drained and, unless otherwise indicated, the muscle was washed 3 times each with 30 ml of Tyrode's solution from a reservoir in the water bath. Stock test solutions were prepared in the required amount of Tyrode's solution, and if necessary, the pH was brought to 7.2 with sodium bicarbonate.

Tryptic hydrolyses of poly-L-lysyl histamine in 0.22 M collidine buffer at pH 7.6 and 26°C were patterned like those described by Waley and Watson(9). The reaction mixtures contained 48 μg trypsin and 20 mg poly-L-lysyl histamine hydrochloride in the collidine buffer. Ten μl aliquots of the reaction were spotted on Whatman 1 paper at zero time, and at 5, 10, 20, 40, 80, and 1,000 minutes after initiation of the reaction. Enzyme and substrate blanks were spotted at the end of each experiment. The enzymatic reactions were terminated on paper by applying 2N acetic acid to the spots. After addition of lysine and histamine markers, the papers were developed 15 hours in ascending fashion in n-butanol-pyridine-water (1:1:1), and the dried papers were sprayed with the imidazole reagent of Block *et al.*(18). In addition, distribution of lysine peptides with and without histamine residues was determined from identical chromatograms containing spots from a 1,000 minute reaction mixture. One chromatogram was sprayed with ninhydrin reagent and the other with imidazole reagent.

Results. The tracings in Fig. 1 indicate that L-lysine (1:2,000) failed to induce contraction of the freshly excised guinea pig ileum, whereas polylysine containing 28 lysyl residues (1:2,000) stimulated the ileum to contract. There was only a slight lag in the response of the muscle to the polymer compared to its immediate response to histamine (1:30,000,000) suggesting that the site of action of the polymer was not hindered. Other isolated segments of ileum contracted in the presence of synthetic L-lysine polypeptides prepared with anhydride to initiator ratios of 10, 40, and 320. The muscles were generally refractory to a second addition of polylysine to the bath but were always sensitive to subsequent additions of histamine. In exceptional cases it was possible to demonstrate (Fig. 2), a very weak response to a second challenge with polylysine. Protamine (1:2,000), although much less effective than polylysine, also stimulated the ileum to contract (Fig. 3). A subsequent addition of polylysine to the bath, however, had no effect on the ileum. Also, polylysine was ineffective after a second histamine response indicating the polymer was not displacing non-specifically bound exogenous histamine. These data indicate polylysine *per se* was not mediating the contraction but was stimulating the release of an active material. The first contact with polylysine presumably depleted the available contractant and subsequent additions of the polymer were ineffective.

Benadryl (1:10,000,000) completely prevented the intestinal response to polylysine, thus implicating endogenous histamine as the contractile agent (Fig. 4). Responses to the synthetic L-lysine polypeptides were obtained only with freshly excised ileum, and there was variation in the magnitude of the contraction. When the muscles were maintained in aerated Tyrode's solution for periods exceeding ½ hour the response to polylysine, but not to histamine, lessened and in most cases became almost nil.

Polylysyl histamine (1:6,000) also induced a contraction of the isolated guinea pig ileum which was blocked by Benadryl. The polylysine moiety alone at this concentration would not produce a contraction of the observed magnitude, and the same muscle responded to a second addition of the polylysyl histamine. These 2 factors preclude activity due to the polylysine portion of the polylysyl histamine and suggest that the response may

FIG. 1. Effect of polylysine on isolated guinea pig ileum.
FIG. 2. Effect of polylysine on isolated guinea pig ileum.
FIG. 3. Effect of protamine on isolated guinea pig ileum.
FIG. 4. Blocking of polylysine action on isolated guinea pig ileum with Benadryl.

be due to traces of free histamine in the preparation.

Polylysyl histamine, as a model substrate, offered an opportunity to study the effects of trypsin on the C-terminal lysyl-histamine bond and further to study the proteolytic theory of histamine release(22). Chromatograms of the tryptic hydrolyzate sprayed with the imidazole reagent clearly showed the lysine polymer was readily hydrolyzed within 5 minutes after initiation of the enzymatic reaction. As the reaction proceeded, 3 imidazole-positive spots became the primary reaction products, and the spot at the origin decreased until it was completely gone at the end of 1,000 minutes. Only a very small trace of the bound histamine, however, was released during the reaction. The chromatograms of the 1,000 minute reaction mixture sprayed with imidazole reagent indicated 3 spots corresponding to a faint trace of histamine with an R_f of 0.44 and 2 major components, probably dilysyl and trilysyl histamine(9) with R_f values of 0.38 and 0.24, respectively. The ninhydrin-treated chromatograms showed the same pattern in addition to large quantities of lysine peptide fragments close to the origin. The results indicate that the lysyl-histamine bond was resistant to tryptic hydrolysis.

Discussion. The contractile response of freshly excised segments of guinea pig ileum to polylysine and protamine and the subsequent refractoriness of the muscle to the polypeptides indicate that the contraction is not due to a direct action of the polymers. The failure of an equivalent amount of L-lysine to induce a contraction suggests that polylysine activity may be due to its basic polyelectrolyte character. There was only a slight lag in response of the muscle to the polymers, as compared to the immediate response to histamine. This small delay and the observation that the muscle is refractory to a second stimulation by the polypeptide indicates that polylysine released some loosely bound substance which in turn induced the contraction. The first addition of polylysine depletes the available contractant, and subsequent additions of polymer are then ineffective. Blocking of the contractile response by Benadryl indicates that loosely bound histamine or similar substances mediates the contraction. Boreus and Chakravarty(23) have recently correlated release of histamine during the antigen-antibody reaction in sensitized guinea pig tissues with disappearance of mast cells. In the jejunum, however, the mast cells did not disrupt significantly, and only a maximum of 3% of total histamine was released. Perhaps only those mast cells near the surface of the intestine are available for histamine release. Assuming that the response to histamine was linear and that concentration of the histamine in the tissues was of the same order of magnitude as those found by Boreus and Chakravarty(23) [10.9 to 14.7 μg/g] an approximation can be made of the quantity of contractant released calculated as per cent of total histamine. Figures which give maximal calculated values indicate that only 4 to 5% of the histamine in the intestine was released in the present studies. The low release of histamine by the intestine may reflect the impermeability or great reactivity of the intestine toward polylysine. These observations are compatible with either the ion exchange(24) or the lecithinase activation (25) concepts of histamine release.

When the muscles were maintained in aerated Tyrode's solution for periods of time exceeding ½ hour the response to polylysine, but not to histamine, lessened considerably. Responses to the synthetic polypeptides were obtained only with freshly excised ileum. The decreased response with time might reflect a slow release of that portion of endogenous histamine which can be rapidly displaced by the polylysine.

Paton(26) has suggested that basic histamine liberators might release endogenous histamine in the gut to stimulate intestinal motility. Perhaps as proteins are hydrolyzed in the gastrointestinal tract, basic peptides similar in their action to polylysine mediate the release of small amounts of endogenous histamine which in turn increases intestinal motility.

Rocha e Silva(27) tested a series of acylated histamines for antihistaminic activity on the isolated guinea pig ileum and demon-

strated that blocking the primary amino group but leaving the imidazole ring free for binding reduced histamine activity and resulted in compounds with weak antihistaminic activity. The concentration of polylysyl histamine required for contraction showed that coupling the histamine to the peptide also greatly reduced the activity of the histamine.

Since trypsin was found to induce contraction of the isolated guinea pig ileum(28) and to release histamine from rabbit platelets (29), Rocha e Silva postulated that endogenous histamine was bound to the carboxyl group of a lysine or arginine residue forming an amide bond with the primary β-ethyl amino group of histamine. Histamine was thought to be released by some proteolytic enzyme with the specificity of trypsin which was activated during anaphylaxis(22). Although the proteolytic theory has generally been displaced in favor of other mechanisms (24,25), the action of trypsin on the lysyl-histamine bond has not been previously experimentally tested. Paper chromatographic studies of the action of trypsin on polylysyl histamine showed that after 16 hours only trace quantities of histamine with an R_f of 0.44 were released from the polypeptide. The bulk of the imidazole residues remained in 2 spots with R_f values of 0.38 and 0.24. Based on the work of Waley and Watson(9), these unknown spots should correspond to lysyl-histamine peptides. A ninhydrin spray revealed the same pattern and other lysine peptides close to the origin. These data indicate that very little of the covalently bound histamine is released by trypsin and are incompatible with the proteolytic theory of histamine release. Perhaps higher levels of trypsin would attack the lysyl-histamine bond as in the case with lysyl amide(30); however, higher levels of trypsin would not be consistent with the concentrations used in biological experiments(28,29,31). Furthermore, based on chemical manipulations of the polymer, there is no unique lability of the lysyl-histamine bond which was once suggested (32) as due to the amplitude of the peptide chain. These data furnish direct evidence that the lysyl-histamine bond is refractory to trypsin hydrolysis, and that adjacent lysyl-lysyl bonds are much more easily split by trypsin.

Summary. L-lysine polypeptides stimulated contraction of freshly excised guinea pig ileum. Ileal segments were refractory to a second challenge of polylysine, and the antihistamine, Benadryl, blocked the action of the polypeptide. Consequently, the contraction was attributed to release of endogenous histamine or a histamine-like substance which was displaced by the basic polypeptide. Polylysyl histamine was synthesized by initiation of polymerization of ϵ-carbobenzoxy-N-carboxy-L-lysine anhydride with histamine. Although polylysyl histamine was readily hydrolyzed by trypsin, the primary products were lysyl histamine peptides which indicated that the histamine-lysine bond was not hydrolyzed by trypsin. The relationship of these data to the proteolytic theory of histamine release was discussed.

1. Burger, W. C., Stahmann, M. A., *Arch. Biochem. Biophys.,* 1952, v39, 27.

2. Katchalski, E., Bichowsky-Slomnicki, L., Volcani, B. E., *Biochem. J.,* 1953, v55, 671.

3. Burger, W. C., Stahmann, M. A., *J. Biol. Chem.,* 1951, v193, 13.

4. Rubini, J. R., Stahmann, M. A., Rasmussen, A. F., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 659.

5. Katchalsky, A., Danon, D., Nevo, A., *Biochim. et Biophys. Acta,* 1959, v33, 120.

6. Katchalski, E., Berger, A., Neumann, H., *Nature,* 1954, v173, 998.

7. Ginsburg, I., Vries, A. de, Katchalski, E., *Science,* 1952, v116, 15.

8. Bienzunski, N., Shafrir, E., Vries, A. de, Katchalski, E., *Biochem. J.,* 1955, v59, 55.

9. Waley, S. G., Watson, J., *ibid.,* 1953, v55, 328.

10. Tsuyuki, E., Tsuyuki, H., Stahmann, M. A., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 318.

11. Richardson, T., Hodgett, J., Linder, A., Stahmann, M. A., *ibid.,* 1959, v101, 382.

12. Buchanan-Davidson, D. J., Stahmann, M. A., Lapresle, C., Grabar, P., *J. Immunol.,* 1959, v83, 552.

13. Stahmann, M. A., Tsuyuki, H., Weinke, K., Lapresle, E., Grabar, P., *Compt. rend. Acad.,* 1955, v241, 1528.

14. Stein, O., Vries, A. de, Katchalski, E., *Arch. intern. pharmacodynamie,* 1956, v57, 243.

15. Garcia-Arocha, J., Ashwin, J. G., Grossberg, A. L., *XIXth Internat. Physiol. Congress,* Abst. Communications, Montreal, Canada, 1954, p 378.

16. Becker, R. R., Stahmann, M. A., *J. Am. Chem. Soc.,* 1952, v74, 38.

17. Ben-Ishai, D., Berger, A., *J. Org. Chem.,* 1952, v17, 1564.

18. Block, R. J., LeStrang, R., Zweig, G., *Paper Chromatography,* 1st edit., Academic Press Inc., New York, 1952, 62-64.

19. Jorpes, E., *Biochem. J.,* 1932, v26, 1507.

20. Code, C. F., McIntire, F. C., in *Methods of Biochemical Analysis,* 1956, vIII, 62.

21. Coulson, E. J., *J. Allergy,* 1953, v24, 458.

22. Rocha e Silva, M., *Histamine, Its Role in Anaphylaxis and Allergy,* 1955, C. C. Thomas Co., Springfield, Ill.

23. Boreus, L. O., Chakravarty, N., *Acta Physiol. Scand.,* 1960, v48, 315.

24. MacIntosh, F. C., Paton, W. D. M., *J. Physiol.,* 1949, v109, 190.

25. Hogberg, B., Uvnas, B., *Acta Physiol. Scand.,* 1957, v41, 345.

26. Paton, W. D. M., *Pharmacol. Rev.,* 1957, v9, 269.

27. Rocha e Silva, M., *J. Pharm. Exp. Ther.,* 1944, v80, 399.

28. ———, *Arquiv. inst. biol. (Sao Paulo),* 1939, v10, 93.

29. Dragstedt, C. A., Rocha e Silva, M., PROC. SOC. EXP. BIOL. AND MED., 1941, v47, 420.

30. Waley, S. G., Watson, J., *Biochem. J.,* 1954, v57, 529.

31. McIntire, R. C., Roth, L. W., Sproull, M., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 605.

32. Rocha e Silva, M., *J. Pharmacol. Exp. Ther.,* 1943, v77, 198.

Received February 7, 1961. P.S.E.B.M., 1961, v107.

A Non-Competitive β-Glucuronidase Inhibitor in Rabbit Urine.* (26628)

GAYLORD M. CONZELMAN, JR., AND DANIEL W. CROUT
(Introduced by L. H. Schmidt)
The Christ Hospital Institute of Medical Research, Cincinnati, Ohio

It has been suggested by Boyland(1) that urinary glucuronidase may be an important factor in induction of bladder cancer by certain aromatic amines. According to this concept, non-carcinogenic glucuronic acid conjugates of 2-amino-1-naphthol might be hydrolyzed by glucuronidase in the bladder to yield the carcinogenic 2-amino-1-naphthol (2).

In an investigation of the relationship of 2-naphthylamine metabolism to bladder tumor induction, urinary β-glucuronidase has been assayed in various species by the method of Boyland *et al.*(3). These determinations revealed marked variations in β-glucuronidase levels of various species. An unexpected finding was the apparent absence of this enzyme in urine obtained from several kinds of rabbits(4). Subsequent experiments showed, however, that rabbit urine did contain β-glucuronidase and that the enzyme could be precipitated from urine with ammonium sulfate. The failure to demonstrate β-glucuronidase activity in whole urine was due to the presence of inhibitory materials. Similar investigations indicated that glucuronidase inhibitors were also present in urine of man, dog, rhesus monkey, mouse and rat(4). These observations confirm and extend the findings of Abul-Fadl(5) and Lewis and Plaice(6) who demonstrated β-glucuronidase inhibitors in human urines. The present report deals with the partial characterization of the β-glucuronidase inhibitor(s) in rabbit urine.

Materials and methods. Two male and 2 female rabbits, inbred animals from a tuberculosis-resistant race,† were used for this study. They were maintained on a diet of Purina Rabbit Pellets with water *ad libitum.*

During experimental periods the animals were housed in metabolism cages equipped with stainless steel funnels; urine was collected into iced vessels, without preservative,

* This project was supported in part by a gift from the Jacob G. Schmidlapp Memorial Fund.

† We are indebted to Dr. Max Lurie, Henry Phipps Inst. for the gift of these animals.

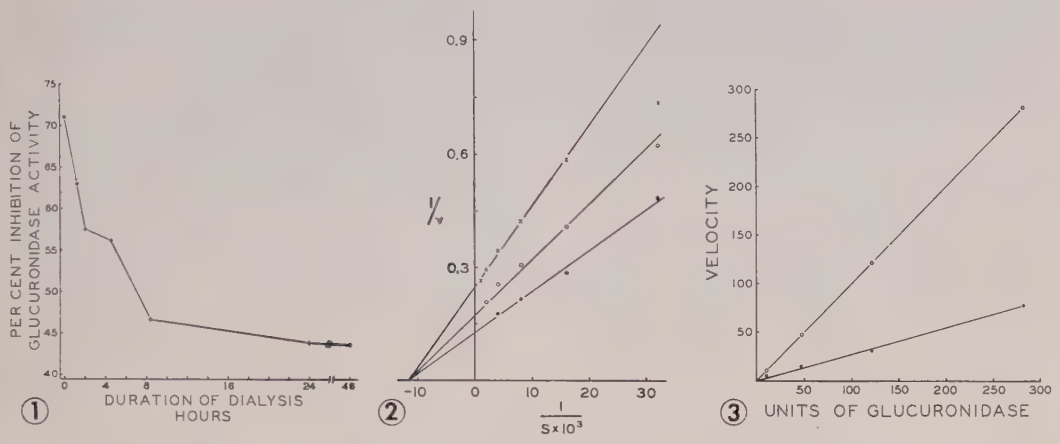


FIG. 1. Reduction in glucuronidase-inhibitor content of rabbit urine after varying periods of dialysis.

FIG. 2. Non-competitive inhibition of β-glucuronidase by rabbit urine dialysate. Reciprocal of initial velocity is plotted against reciprocal of phenolphthalein glucuronide concentration. Velocity expressed as micrograms of phenolphthalein liberated per ml per hour. Reaction mixtures contained 0.2 ml 0.2 M acetate buffer (pH 4.5), 0.2 ml enzyme solution and phenolphthalein glucuronic acid in concentrations varying from 3.125×10^{-5} to 1.0×10^{-3} M in a total vol. of 0.6 ml. Curve A (●——●), obtained in absence in inhibitor; Curve B (○——○), obtained in presence of 0.2 ml 1:8 dilution rabbit urine dialysate; Curve C (×——×), obtained in presence of 0.2 ml of 1:2 dilution rabbit urine dialysate.

FIG. 3. Relationship of reaction velocity (v) to β-glucuronidase concentration in presence and absence of urinary inhibitor. Velocity expressed as micrograms of phenolphthalein liberated per ml per hour from 0.4 mM phenolphthalein glucuronide at pH 4.5. Inhibitor concentration: ○——○, nil; ●——●, 1:3 dilution of rabbit urine dialysate.

for 24-hour periods. This chilled urine was used within a few hours after collection.

β-glucuronidase activity was determined by a slight modification of the method of Boyland *et al.*(3), using phenolphthalein glucuronic acid‡ as substrate and either *Ketodase* or Worthington β-glucuronidase as enzyme source. Unless otherwise indicated, the reaction was carried out at 37°C in 0.2 M acetate buffer, pH 4.5, with appropriate control and reagent blanks. The action of the enzyme was halted by addition of 10% sodium carbonate solution and glycine buffer, pH 10.6, and the reaction mixture clarified by centrifugation at 1600 × g for 15 minutes. The amount of phenolphthalein liberated was determined in a Klett-Summerson Colorimeter, using a No. 54 filter.

The mucoprotein fraction of urine was isolated from pooled 24-hour specimens of rabbit urine by the method of Anderson and Maclagan(7) and showed a typical diphenylamine reaction. It was dissolved in either acetate buffer, pH 4.5, or in a "synthetic urine," which contained inorganic ions and organic compounds at concentrations corresponding to those found in normal urine.

For the dialysis studies, urine samples were placed in cellulose tubing and were dialyzed at 4°C for 24 hours against running distilled water.

Results and discussion. The anti-glucuronidase activity of rabbit urine can be reduced but not eliminated by dialysis against cold running distilled water (Fig. 1). The loss of diffusible inhibitory material proceeds fairly rapidly during the first 8 hours of dialysis, then slows down markedly. The non-dialyzable fraction remaining after 48 hours of dialysis represents 60% of the total inhibitor present in urine.

Subjecting rabbit urine which had been dialyzed for 24 hours to autoclaving for 15 min at 15 lb pressure had no effect on the activity of the non-dialyzable inhibitor, indicating that it was heat-stable, but the ash from urine dialysates contained no glucuronidase inhibitors. This inhibitor therefore appears to bear a resemblance to the non-dialyzable, heat-stable, protein-like inhibitor(s) found

‡ Sigma Chemical Co.

TABLE I. Effect of Hydrogen Ion Concentration on Activity of Urinary Glucuronidase Inhibitor(s).

pH	% inhibition	pH	% inhibition
3.0	34.4	5.5	51.4
3.5	42.2	6.0	42.2
4.0	44.2	6.5	6.7
4.5	45.9	7.0	.0
5.0	47.2		

in plasma by Fishman(8), and to an inhibitor(s) extracted from urine by Abul-Fadl (5). However, isolation of the mucoprotein fraction from rabbit urine by benzoic acid precipitation(5) yielded a product which was devoid of anti-glucuronidase activity.

Assay of the non-dialyzable, heat-stable inhibitor in rabbit urine at various pH levels indicates that it is active over a pH range from as low as 3.0 to approximately 6.0, with a maximum at pH 5.5 (Table I). Furthermore, the results obtained in kinetic studies agree closely with those expected for pure non-competitive inhibition(9). This was concluded from the following experiments.

The reaction velocity of β-glucuronidase was determined without inhibitor and in the presence of rabbit urine dialysate at 2 concentrations (1:2 and 1:8). When the reciprocals of the reaction velocities (1/v) are plotted against the reciprocals of substrate concentration (1/S), curve A (control) intercepts the 1/v axis at 0.13, corresponding to the liberation of 3.9 γ phenolphthalein/ml/hr. The corresponding intercepts for curve B (1:8 urine dialysate) and curve C (1:2 urine dialysate) were 0.18 and 0.25, equivalent to maximum velocities of 2.8 and 2.0 γ/ml/hr. The substrate concentration giving half maximum velocity (Km), which was unchanged by the presence of inhibitor, was 8.7×10^{-5} M.

To exclude the possibility of "pseudo-irreversible" inhibition, the test of Ackerman and Potter(10) was applied (Fig. 3). The resulting curves, representing velocity of hydrolysis as a function of glucuronidase concentration, in presence and absence of a constant amount of inhibitor, were indicative of reversible inhibition.

These investigations indicate that the failure of rabbit urine to hydrolyze glucuronic acid conjugates is due not to absence of glucuronidase, but to the presence of a glucuronidase inhibitor(s) which functions in a non-competitive fashion. They suggest that the resistance of rabbits to bladder carcinogens such as 2-naphthylamine may be attributable to inhibition of urinary β-glucuronidase, and the consequent inadequate hydrolysis of 2-amino-1-naphthyl glucuronide to the carcinogen, 2-amino-1-naphthol.

Summary. A heat-stable, non-dialyzable inhibitor of β-glucuronidase is present in rabbit urine. It is not associated with the mucoprotein fraction of urine. Kinetic studies indicate the substance(s) functions as a non-competitive inhibitor, which combines reversibly with the enzyme.

1. Boyland, E., *Bull. Soc. Chim. Biol.,* 1956, v38, 827.

2. ———, *Brit. Med. Bull.,* 1958, v14, 153.

3. ———, Gasson, J. E., Williams, D. C., *Brit. J. Cancer,* 1957, v11, 120.

4. Conzelman, G. M., Jr., Crout, D. W., Unpublished studies.

5. Abul-Fadl, M. A. M., *Biochem. J.,* 1957, v65, 16.

6. Lewis, F. J. W., Plaice, C. H. J., *Brit. J. Cancer,* 1960, v14, 106.

7. Anderson, A. J., Maclagan, N. F., *Biochem. J.,* 1955, v59, 638.

8. Fishman, W. H., Ciba Foundation *Colloquia on Endocrinology,* v1, Steroid Hormones and Tumor Growth and Steroid Hormones and Enzymes, The Blakiston Co., Philadelphia, 1952.

9. Lineweaver, H., Burk, D., *J. Am. Chem. Soc.,* 1934, v56, 658.

10. Ackerman, W. W., Potter, V. R., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 1.

Received March 6, 1961. P.S.E.B.M., 1961, v107.

Effects of Tolbutamide on Cardiovascular Function and Myocardial Metabolism of Intact Dogs.* (26629)

BENJAMIN WITTELS AND DONALD B. HACKEL
Department of Pathology, Western Reserve University School of Medicine at Cleveland Metropolitan General Hospital, Cleveland, Ohio; and Department of Pathology, Duke University Medical Center, Durham, N. C.

The effects of tolbutamide on peripheral glucose utilization and on hepatic glucose output have been investigated extensively, but remain subjects of considerable controversy(1,2). Interpretation of much of the data is limited by lack of information on rates of blood flow in conjunction with measurement of glucose arteriovenous differences (3) and by certain assumptions in radioactive tracer methods(4). Technics developed to study myocardial metabolism in the intact dog are free of these disadvantages and have been applied in the present work to elucidate the action of tolbutamide on the heart.

Materials and methods. The experimental model employed has been described in detail (5). Eight well-nourished mongrel dogs were deprived of food but not of water overnight, (approximately 18 hours). They were then anesthetized with morphine, 3 mg/kg, subcutaneously, followed in 30 minutes by a 50/50 mixture of Nembutal and Dial-urethane,† 0.25 cc/kg, intravenously. The trachea was intubated and the coronary sinus, aorta and inferior vena cava were catheterized *via* a jugular vein, femoral artery and femoral vein respectively. During catheterization the animals were given heparin, 5 mg/kg, intravenously, aqueous penicillin, 100,000 units, and streptomycin, 1 g, intramuscularly.

The following were determined: cardiac output (Fick principle with O_2); coronary blood flow (nitrous oxide desaturation method); aortic and coronary sinus whole blood concentrations of glucose, pyruvate, lactate, O_2 and CO_2; aortic and coronary sinus plasma concentrations of Na^+ and K^+; and aortic blood pH. Systemic blood pressure was registered on a water manometer connected to the femoral artery catheter. Heart rate was determined on electrocardiograms recorded with a Sanborn Viso-Cardiette.

Immediately after the blood samples and recordings were obtained, tolbutamide (Orinase),† 40 mg/kg, was injected rapidly into the femoral vein catheter. One hour later the sampling and recording were repeated. This interval was selected because preliminary observations revealed that the maximal change in the arterial blood glucose concentration was usually affected by this time. All animals recovered from the experiments.

Results. Significantly decreased arterial blood concentrations of glucose developed by one hour after tolbutamide administration (Table I). Despite this fall, myocardial extraction (coronary arteriovenous difference) and utilization (coronary arteriovenous difference $\times$ coronary blood flow) of glucose were fully maintained. This continued ability of the myocardium to obtain glucose in undiminished amounts at the lowered arterial concentrations is shown by the striking elevation of the percent extraction of glucose (coronary arteriovenous difference/arterial concentration $\times$ 100). No significant modifications were produced by tolbutamide in the arterial concentrations and myocardial usage of pyruvate and lactate. Arterial concentrations of Na^+ (control 148.3 $\pm$ 1.0 mEq/l, treated 150.1 $\pm$ 1.4 mEq/l) and of K^+ (control 4.3 $\pm$ .2 mEq/l, treated 4.4 $\pm$ .3 mEq/l) did not change significantly, and neither before nor after tolbutamide administration was there a significant coronary arteriovenous difference of these substances.

Oxygen concentration and saturation of the arterial blood remained stable and there was no significant change in oxygen con-

* Supported by grants from Cleveland Area Heart Soc. and U. S. Public Health Service.

† The Dial-urethane was generously contributed by Ciba Pharmaceutical Products, Inc., Summit, N. J., and tolbutamide (Orinase) by Upjohn Co., Kalamazoo, Mich.

TABLE I. Metabolic Findings Before (Control) and One Hour After (Treated) Administration of Tolbutamide (Mean ± S. E. of Mean).

		Arterial concentration, mg (or vol) %	Myocardial extraction,* mg (or vol) %	% extraction†	Myocardial utilization,‡ mg (or vol)/100 g/min.
Glucose	Control	74.1 ± 2.3	10.6 ± 1.6	14.5 ± 2.3	9.4 ± 1.4
	Treated	51.6 ± 4.5 §	14.0 ± 1.9	26.9 ± 2.6§	11.4 ± 1.7
Pyruvate	Control	1.10 ± .12	.58 ± .10	50.6 ± 4.1	.52 ± .10
	Treated	1.12 ± .09	.72 ± .06	65.3 ± 4.1	.58 ± .04
Lactate	Control	8.0 ± 1.1	3.6 ± .7	43.6 ± 4.7	3.1 ± .6
	Treated	10.0 ± 1.1	4.9 ± .6	50.7 ± 4.4	4.1 ± .6
Oxygen	Control	19.1 ± .4	12.5 ± .6	66.3 ± 3.3	11.3 ± .7
	Treated	20.2 ± .5	14.5 ± .6	71.7 ± 3.0	11.7 ± .5

* Coronary arteriovenous difference.
† Coronary arteriovenous difference/arterial concentration × 100.
‡ Coronary arteriovenous difference × coronary blood flow.
§ Indicates a significant difference from control value ($p < .01$).

sumption by the heart or in its respiratory quotient (.84 ± .06 to .91 ± .03). Although arterial carbon dioxide content decreased (45.2 ± .9 to 41.3 ± 1.2 volumes %) ($p < .05 > .01$), arterial pH was not affected (7.20 ± .01 to 7.23 ± .08).

No significant alterations occurred in the cardiovascular dynamics under investigation (Table II).

Discussion. During tolbutamide-induced hypoglycemia in the intact, anesthetized dog, the heart was capable of extracting blood glucose in amounts equivalent to those obtained during the normoglycemic state. This ability is expressed by the significantly increased percent extraction of glucose. In contrast, depression of arterial glucose concentration to comparable levels by starvation(5) or by diphtheria toxin is associated with a marked reduction in glucose extraction by the myocardium. These observations may be construed to indicate that tolbutamide, directly or otherwise, facilitates the passage of glucose from the blood into the myocardial cell even when the concentration of arterial glucose is diminished.

TABLE II. Hemodynamic Findings Before (Control) and One Hour After (Treated) Administration of Tolbutamide (Mean ± S.E. of Mean).

	Control	Treated
Aortic blood pressure, mm Hg	116.5 ± 4.2	120.9 ± 4.0
Heart rate, beats/min.	63.1 ± 4.0	83.1 ±11.4
Coronary blood flow, ml/100 g/min.	89.0 ± 2.4	81.0 ± 3.4
Cardiac index, l/meters²/min.	2.6 ± .4	2.3 ± .3
Left ventricular work, kg meters/min.	3.9 ± 1.0	3.5 ± .5
Left ventricular efficiency, %	15.0 ± 3.2	12.3 ± 1.7
Peripheral vascular resistance (arbitrary units)	2.61± .30	3.22± .55
Coronary vascular resistance (arbitrary units)	78.9 ± 3.6	91.0 ± 5.5

Note: There are no statistically significant differences between control and treated groups.

That tolbutamide increases peripheral utilization of glucose has been asserted(6,7) but also denied(8,9). Under the conditions prevailing in our dogs, the myocardium maintained but did not increase its usage of glucose. It has been demonstrated that the amount of glucose extracted by the heart is directly related to arterial glucose concentrations at values exceeding the myocardial threshold(5). Even when entrance of glucose into the cell is expedited by tolbutamide, the availability of the substrate may be pertinent in determining the degree of its utilization. In contrast to its effects upon the metabolism of glucose, tolbutamide failed to influence significantly that of the other substrates which were studied.

Numerous investigations have indicated that the metabolic activities of tolbutamide are consequent to the release of endogenous

insulin(10,11,12,13). This conclusion is also supported by the notable similarity of the effects of tolbutamide to those of injected insulin(14) upon cardiac carbohydrate metabolism.

Summary. Tolbutamide, 40 mg/kg, was rapidly administered into the femoral vein of intact, anesthetized dogs, deprived of food overnight. One hour later the following were observed: a) a significant decrease in arterial concentration of glucose; b) a significant elevation in *percent* of blood glucose extracted by the myocardium without any significant change in *absolute* amount extracted or *total* amount utilized; c) no significant change in arterial concentration or myocardial extraction and utilization of pyruvate, lactate, oxygen, Na^+ or K^+; d) no significant deviation in cardiovascular dynamics including coronary blood flow and cardiac index.

1. Stadie, W. C., *Diabetes,* 1958, v7, 61.
2. Ashmore, J., *Metabolism,* 1959, v8, 469.
3. Shoemaker, W. C., Mahler, R., Ashmore, J., *ibid.,* 1959, v8, 494.
4. Stetten, D., Jr., *ibid.,* 1959, v8, 559.
5. Goodale, W. T., Hackel, D. B., *Circulation Research,* 1953, v1, 509.
6. Goetz, F. C., Gilbertsen, A. S., Josephson, V., *Metabolism,* 1956, v5, 788.
7. Miller, W. L., Jr., Krake, J. J., Vanderbrook, M. J., *J. Pharm. Exp. Therap.,* 1957, v119, 513.
8. Renold, A. E., Winegrad, A. I., Froesch, E. R., Thorn, G. W., *Metabolism,* 1956, v5, 757.
9. Purnell, R., Arai, Y., Pratt, E., Hlad. C., Jr., Elrick, H., *ibid.,* 1956, v5, 778.
10. Houssay, B. A., Penkos, J. C., Urgoiti, E., Teodosio, N., Apelbaum, J., Bowkett, J., *Ann. N. Y. Acad. Sci.,* 1957, v71, 25.
11. Goetz, F. C., Egdahl, R. H., *Fed. Proc.,* 1958, v17, 55.
12. Colwell, A. R., Jr., Colwell, J. A., *J. Lab. & Clin. Med.,* 1959, v53, 376.
13. Yalow, R. S., Black, H., Villazon, M., Berson, S. A., *Diabetes,* 1960, v9, 356.
14. Hackel, D. B., *Am. J. Physiol.,* 1960, v199, 1135.

Received March 10, 1961. P.S.E.B.M., 1961, v107.

Changes in Phosphorus Metabolism of the Gastrocnemius Muscle in Aging White Rats.* (26630)

MORRIS ROCKSTEIN AND KARL F. BRANDT
Department of Physiology, New York University Medical Center, New York City

In pursuit of evidence for the corollary to Sumner's classical statement(1) that "life is an orderly function of enzymes" *viz.* that senescence in structures exhibiting anatomical and functional decline with age should be reflected by corresponding alterations in activity of significant cell catalysts, a long-range study has been under way in this laboratory of the changes in enzymes related to metabolism of high-energy organophosphorus compounds in skeletal muscle of aging rats. Potter, *et al.*(2) reported a marked rise in adenosine triphosphatase (ATP-ase) activity in the skeletal muscle of the immediately postpartum rat. Moog(3) and Robinson(4) in developing chick muscle and De Villafranca(5) in developing rat muscle also found a corresponding rise in ATP-ase activity with development of fibrillar substance. As for the organophosphorus compounds (including the corresponding substrate ATP) there are conflicting reports for distribution of acid-soluble phosphorus compounds with age in human(6) and in rat skeletal muscle (7). A recent study by Rockstein, involving both the enzyme system(8) and the related adenine nucleotides(9), important in the energizing of muscular contraction related to flight in insects, revealed a marked decline in the Mg-activated ATP-ase and other enzymes and a concomitant accumulation of

* Supported in part by a grant from U. S. Public Health Service. The authors are appreciative of the early participation and preliminary enzyme determinations, in connection with this study, by Dr. Filomena Boffoli.

ATP, with decline and loss in the flight ability in males. This suggested a similar study on changes in enzyme activity and related adenine nucleotides in skeletal muscle of aging rats, particularly in a strain showing marked loss in muscular function in senile animals. This report summarizes the results of over 3 years of such a study.

Materials and methods. Tissue preparation. Male rats of the Sprague-Dawley strain (from the Hormone Assay Laboratories) were bred, reared and maintained in temperature-controlled animal quarters and fed *ad libitum* on a standardized commercial laboratory diet. Each animal was examined and weighed once weekly, beginning with the end of the third month. For each analysis an animal of known age was sacrificed by rapid separation of the brain from the spinal cord and rapid excision of the tissue under study. The gastrocnemius muscle was removed, *in toto,* cut transversely into 2 or 3 approximately equal sections according to size, then rinsed in ice-cold pH 7.4 Tris (Sigma) buffer solution, and dried briefly on paper towelling before being weighed. The sections were then plunged into liquid air and fast-frozen for 10 minutes before being transferred to capped polyethylene vials and stored in a freezer at −30°C until studied. For each enzyme (or substrate) determination, the frozen sample of muscle was cooled further in liquid air and then crushed into a powdery consistency in a previously cooled Elvehjem-Potter stainless steel crusher. The crushed tissue was then transferred quantitatively to an Elvehjem-Potter glass homogenizer cup containing 2 ml ice cold 7.4 Tris buffer and homogenized mechanically with a Teflon pestle for 2 minutes. The homogenate was transferred quantitatively (including rinses of pestle and homogenizer cup with ice-cold 7.4 Tris buffer) into a cold centrifuge tube made to a final volume of 5 ml of buffer and allowed to stand in an ice bath for 20 minutes at zero°C in order to extract water-soluble enzymes. After being centrifuged for 10 minutes at 2500 rpm (970 g) in a refrigerated centrifuge at zero° C the supernatant liquid was carefully decanted and stored in an ice bath for study of the Mg-activated (water-soluble) enzyme. Where the Ca-activated (actomyosin) enzyme activity was estimated, the centrifugate was resuspended in 7 ml ice-cold 0.6 *M* K Cl in pH 9.0 Tris buffer solution containing 0.002 *M* Versene (Na tetraversenate) as an "activator" of actomyosin ATP-ase. After elution by standing at zero°C for 20 minutes, the suspension was centrifuged at zero° C for 10 minutes at 2500 rpm and the (high ionic strength salt solution) extractable actomyosin solution decanted and stored cold. This brei was employed in determination of actomyosin (pH 9.0) ATP-ase activity.

Enzyme assay. To one ml of homogenate in 7.4 Tris buffer solution were added 0.1 ml each of ice-cold 0.03 *M* ATP and of 0.03 *M* Mg Cl_2, each prepared in the 7.4 Tris buffer solution. After addition of a drop of chloroform, the incubation tube was stoppered and mixed by inversion 3 times and transferred to a constant temperature water bath at 37°C. At the end of a 5-minute period required to reach temperature equilibrium with the water bath, a zero-time (control) 0.5 ml sample was removed from the freshly remixed incubation tube into a vial containing 0.2 ml of 20% trichloroacetic (TCA) and the contents thoroughly mixed. Fifteen minutes later, a second 0.5 ml aliquot was similarly removed from the incubation tube into a second vial of 20% TCA; this was the experimental sample. After 5 minutes to permit complete deproteinization, the mixture was filtered through a Whatman 42 (4.25 cm) filter disc folded into a one-inch glass funnel. Enzyme activity was determined in terms of differences between the inorganic phosphate content of 0.2 ml samples of deproteinized experimental and control filtrates. This was determined by the ferrous-sulfate-acid molybdate method of Rockstein and Herron(10). For each tissue preparation (3 per muscle), 5 such pairs of experimentals and controls were made for each pH level indicated. The gastrocnemius muscles from 10 animals were studied for each age group.

Results. Table I shows the changes with age of Mg-activated (pH 7.4) ATP-ase activity for both male Sprague Dawley and

TABLE I. Age Changes in Mg-ATP-ase Activity and Weight of Gastrocnemius Muscle of the Male White Rat.*

Strain	1 Median age (mo)	2 ATP-ase activity	3 Muscle wt (g)	4 Muscle wt/body wt ($\times 10^{-3}$)
Sprague-Dawley	9.5 (7-12)	.69 ($\pm$ .13)	4.58	11.93
	16.5 (13-20)	.51 ($\pm$ .18)	4.63	9.47
	26.0 (23-28)	.24 ($\pm$ .07)	2.45	6.69
CFN	8.0 (5-12)	.79 ($\pm$ .15)	4.10	8.92
	17.5 (14-18)	.58 ($\pm$ .10)	4.89	9.34
	26.0 (24-33)	.37 ($\pm$ .05)	4.55	7.91

* Values in parentheses, column 1, show actual ranges of ages for the various median age groups. Data in columns 2 and 3 represent median enzyme and gastrocnemius muscle mass values for 10 animals of each median age group. ATP-ase activity expressed in μg of P released in 15 min., per g of fresh muscle mass. Values in parentheses in column 2 are probable deviations.

CFN, specific pathogen-free, white rats. The latter were offspring (delivered by Caesarean section) from Carworth Farms females and nursed by Nelson, germ-free foster mothers. Median age groups indicated in column 1 are based on age-dependent, weight-growth curves (to be published) and the fact that the somewhat longer-lived CFN animals have a long middle age, during which the body weight remains unchanged, beginning with the 300 days after birth and through the 800th day, in contrast to the shorter-lived Sprague-Dawley males with the shorter middle age of steady weight occurring at 400 to 600 days after birth. Also shown are the total gastrocnemius muscle weights and muscle weight:body weight ratios. In the Sprague-Dawley strain the fall in enzyme activity was very marked, that of senile rats being only about 35% of that of young, growing animals, a fall of 65%. In the old CFN rats, on the other hand, enzyme activity falls about only 50%. Moreover, the total gastrocnemius muscle mass is seen to fall much more markedly than does the absolute body weight, *i.e.*, this ratio falls by 45%. This means that, in the physiologically senile Sprague-Dawley animal, the loss in Mg-activated ATP-ase is even greater on an absolute body weight basis (at least twice that which is expresed on a total muscle weight basis). In the CFN strain, on the other hand, there is virtually no change in muscle to body weight ratios from young to old age and, therefore, the enzyme activity available on a body weight basis to the senile CFN males is considerably more than twice that found in the shorter-lived strain with a shorter constant weight middle age and a longer period of physiological senility.

It is of considerable interest (*See Discussion*) that no changes were found for either strain in the actomyosin (pH 9.0) ATP-ase activity nor in the adenosine triphosphate (ATP) and adenosine monophosphate (AMP) content of the gastrocnemius muscle, with advancing age.

Discussion. Two major considerations are implicit in these data. The first concerns the completely different picture as regards changes in the Mg-activated (pH 7.4) ATP-ase and the actomyosin (pH 9.0) enzyme, with advancing age. Although the actomyosin dephosphorylating system must be considered more directly concerned with the process of contraction in skeletal muscle, it it remarkable that its specific activity remains unchanged in a strain showing pronounced failure in motor function in very old animals. On the other hand, the activity of the Mg-activated enzyme shows a tremendous fall with advancing age in this same strain. This is especially significant inasmuch as De Villafranca (5, *op. cit.*) has also demonstrated conclusively that the Mg-activated ATP-ase shows a pronounced rise in activity during development and maturation of skeletal muscle in the post-partum rat up to about 30 days. De Villafranca suggested that the Mg enzyme may be involved here in protein synthesis during formation of the myofibrillae. However, the Mg-activated en-

zyme system has been studied extensively at the Wenner-Grens Institute by Low *et al.* and other colleagues of Lindberg(11). They suggest that the skeletal muscle Mg-activated ATP-ase serves as a transphosphorylating catalyst between ATP and reduced diaphorase flavin, and is therefore an important enzyme in the complex of accessory reactions involved in the oxidative aspects of phosphorylation. Thus, both maturation of the contractile mechanism, in the case of the *developing* rat, as well as in the gradual failure of motor function in the *senescent* rat, indicate a direct or indirect role of the effectiveness of the phosphorylating mechanism in the myofibrillae at different ages. However, in the case of the rat skeletal muscle, our findings that there is no change in ATP or ADP content on a unit muscle-weight basis despite the marked fall in activity of the Mg-activated ATP-ase, suggest that alternative pathways may be operating in oxidative phosphorylation in aging rat skeletal muscle. This is also evident from the above-reported strain differences. It is interesting to note, for example, that gross manifestations of functional decline are apparent in the strain which shows a muscular dystrophy-like syndrome of the hind limbs; concomitantly, the specific activity of the Mg-activated enzyme in the gastrocnemius muscle falls very markedly and, on an absolute body weight basis, even more drastically. In the CFN strain, on the other hand, with a greater life span, a longer period of middle age, with constant maximum age and only a moderate decline in motor function (with no outward signs of the above-mentioned muscular dystrophy-like syndrome) in extreme old age, this enzyme system, on a *unit muscle mass basis* shows a smaller percentage loss in activity with extreme old age. With the muscle mass to body weight ratio remaining practically unchanged in CFN animals, final enzyme activity in the gastrocnemius muscle of this strain *on an absolute body weight basis* is at least twice as great as that of the Sprague-Dawley animals. Thus, whatever the exact role which this enzyme system may play in the effective utilization of the skeletal muscle components of aging mammals from birth to senility, we have in the Mg-activated ATP-ase system a firm, reproducible biochemical criterion for senescence of skeletal muscle function.

Summary. Changes in the adenine nucleotide dephosphorylating system in the gastrocnemius muscle of aging male rats have been studied. The Mg-activated ATP-ase activity showed a pronounced fall from young to very old (senile) age in both the Sprague-Dawley and the CFN strains. However, in the former strain, old animals, typically exhibiting markedly greater losses in muscle mass and concomitant muscular dystrophy, also showed a greater decline in this enzyme on a unit muscle mass and even more so on an absolute body mass basis, than did the old CFN rats. In both strains, however, actomyosin (pH 9.0) ATP-ase as well as ATP content showed no significant changes from young to very old age. The role of the Mg-activated ATP-ase enzyme in energizing of skeletal muscle contraction and its possible significance in the physiology of senescence are discussed.

1. Sumner, J. B., Myrbäck, K., *The Enzymes,* Academic Press, N. Y., 1950, v1, Pt. I, Ch. I.

2. Potter, V. R., Schneider, W. C., Liebel, G. J., *Cancer Res.,* 1945, v5, 21.

3. Moog, F., *J. Exp. Zool.,* 1947, v105, 209.

4. Robinson, D. S., *Biocheem. J.,* 1952, v52, 621.

5. De Villafranca, G. W., *J. Exp. Zool.,* 1954, v127, 367.

6. Struck, H. C., Visscher, M. B., *Am. J. Physiol.,* 1935, v113, 128.

7. Horvath, S. M., *ibid.,* 1945, v145, 77.

8. Rockstein, M., *J. Gerontol.,* 1956, v11, 282.

9. Rockstein, M., Gutfreund, D. E., *Science,* 1961, accepted for publication.

10. Rockstein, M., Herron, P. W., *Analyt. Chem.,* 1951, v23, 1500.

11. Löw, H., *On the Mechanism of Oxidative Phosphorylation,* Almquist & Wiksells, Uppsala, 1959.

Received March 14, 1961. P.S.E.B.M., 1961, v107.

Blood Tocopherol Values in Normal Human Adults and Incidence of Vitamin E Deficiency. (26631)

PHILIP L. HARRIS, EDWARD G. HARDENBROOK, FREDERICK P. DEAN, ELEANOR R. CUSACK, AND JAMES L. JENSEN*

Biochemistry Laboratories, Distillation Products Industries, Division of Eastman Kodak Co., Rochester, N. Y.

Under controlled experimental conditions, the human requirement for Vit. E varies 6-fold or more, depending on other dietary factors—primarily the content of unsaturated fat(1,2). This discovery has stimulated interest in the Vit. E nutriture of populations of normal subjects on practical diets.

For this reason we present in Table I the results of a survey of blood tocopherol levels made in Rochester, N. Y., as part of a comprehensive study of the influence of multivitamin supplementation on blood levels of Vit. A, C, E, and carotene.

The subjects, presumably in good health and receiving adequate diets, were 120 male and 77 female industrial workers, 17 to 64 years of age. At the beginning of the study, before supplementation, the values ranged from 0.36 to 1.80 mg/100 ml. (For tocopherol analysis, the method of Quaife and Harris was used(3) on blood samples drawn in the morning while the subjects were in a fasting state.) In agreement with the mean values found for other populations (Table II), the mean value was 1.05 ± .32 mg tocopherol/100 ml plasma. Thus the present data confirm the generally accepted conclusion that the normal plasma tocopherol level for humans is very close to 1.0 mg/100 ml.

It is important to recognize, as Edwin *et al.*(21) have pointed out in their critical evaluation of analytical procedures used for measuring tocopherols in animal tissues, that most such values represent total reducing materials rather than tocopherol *per se*. Chromenols, reduced ubiquinones, and other antioxidants as well as tocopherols may be present in blood and other animal tissues; these are measured as tocopherol unless such a step as paper chromatography as recommended by Edwin and coworkers is included in the procedure.

TABLE I. Frequency Distribution of Plasma Tocopherol in Normal Adults (Rochester, N. Y.).

Plasma tocopherol values (mg/100 ml)	No. of persons	Frequency (%)
.30- .39	3	2
.40- .49	10	5
.50- .59	6	3
.60- .69	9	4
.70- .79	14	7
.80- .89	13	7
.90- .99	22	11
1.00-1.09	29	15
1.10-1.19	34	17
1.20-1.29	21	11
1.30-1.39	8	4
1.40-1.49	8	4
1.50-1.59	6	3
1.60-1.69	9	4
1.70-1.79	4	2
1.80-1.89	1	1
	197	100
Mean	1.05 mg ± .32 (S.D.)	

In future nutritional surveys, measuring some physiological function related to the tocopherol nutriture of the body would be even better than measuring tocopherol *per se*. Such a method—measurement of the susceptibility of red blood cells to hemolysis—is now available.† Increased susceptibility of red blood cells to hemolysis is the earliest indication of Vit. E deficiency, as was discovered first in animals(22) and is now known to apply to human infants(23,24) and adults(1,2).

* Present address: Beebe Laboratories, Inc., St. Paul, Minn.

† The test is easy to do, and the results are unequivocal(25). The red blood cells from a few drops of blood are washed, then incubated with dilute hydrogen peroxide. The amount of hemoglobin released by rupture of red blood cells is measured colorimetrically and expressed as a percentage of complete hemolysis. Persons with adequate vit E nutriture have red blood cells that are completely resistant; those low in tocopherol have red blood cells that show a significant degree of hemolysis—over 20%.

TABLE II. Plasma Tocopherol in Normal Humans.

No. of subjects	Locale	Mean	S.D.	Reference
122	Holland	.77	± .35	Engel(4)
14	New York City	.78	± .39	Hillman and Rosner(5)
74	Nashville	.89	± .20	Ferguson *et al.*(6)
20	Italy	.92	± .25	Rindi and Perri(7)
12	New York City	.96	± .33	Wechsler *et al.*(8)
188	St. Louis	.98	± .30	Chieffi and Kirk(9)
116	Hungary	.99	± .25	Kramer(10)
35	Steilacoom (Wash.)	1.04	± .25	Van Bruggen and Straumfjord(11)
70	Birmingham	1.04	± .30	Harris *et al.*(12)
17	Rochester	1.05	± .27	Scrimshaw *et al.*(13)
583	England	1.05	± .23	Leitner *et al.*(14)
197	Rochester	1.05	± .32	Harris *et al.*, present study
10	Durham	1.06	± .06	Darby *et al.*(15)
30	Philadelphia	1.08	± .29	Urbach *et al.*(16)
21	Nashville	1.09	± .17	Lemley *et al.*(17)
13	Rochester	1.20	± .22	Harris and Quaife(18)
30	Boston	1.20	± .22	Postel(19)
23	New Haven	1.23	± .31	Klatskin(20)
1575	Weighted mean* =	1.01	± .24 (S.D.†)	

$$* \text{ Weighted mean} = \frac{\overline{M}_1 n_1 + \overline{M}_2 n_2 + \overline{M}_3 n_3 + \text{etc.}}{N}.$$

$$† \text{ s (stand. dev.)} = \sqrt{\frac{s_1^2 (n-1) + s_2^2 (n-1) + s_3^2 (n-1) + \text{etc.}}{N - K}},$$

where N = No. of individual values (determinations) and K = No. of separate means (studies).

In both children and adults a significant degree of red blood cell hemolysis occurs when serum or plasma tocopherol concentration falls below 0.5 mg/100 ml. Using this relationship, a tentative interpretation with respect to incidence of Vit. E deficiency can be made of the data from the present survey and those previously reported.

The Rochester study shows that 7% of the subjects had values in the deficiency range. Only 3 other surveys gave the distribution in detail; these show that 12% of the Holland subjects, 4% of the Birmingham subjects, and 2% of the British subjects had blood tocopherol values in the deficiency range.

There is not yet enough information available for us to speculate on the diets of the persons having low blood tocopherol values, especially concerning the newly-raised questions: were the α-tocopherol intakes (in contrast to total tocopherol) relatively low, or were the unsaturated fat intakes relatively high? Whichever the case, these data show that a significant number of persons had a tocopherol deficiency symptom. Future nutrition surveys, using the blood hemolysis test, will not only supply interesting comparative data on incidence of tocopherol deficiency—they may also show relationships between dietary tocopherol intake and red blood cell hemolysis test values from which tocopherol requirements (minimum daily requirement under a variety of conditions) can be calculated.

Summary. The mean tocopherol concentration in 197 factory workers in Rochester, N. Y., was 1.05 ± 0.32 mg/100 ml. About 7% of the subjects had less than 0.50 mg tocopherol/100 ml, the level below which red blood cell hemolysis tests become positive, indicating Vit. E deficiency.

1. Horwitt, M. K., *Am. J. Clin. Nutrition,* 1960, v8, 451.

2. ———, *Borden's Rev. Nutrition Research,* 1961, v22, 1.

3. Quaife, M. L., Harris, P. L., *J. Biol. Chem.,* 1944, v156, 499.

4. Engel, C., *N. Y. Acad. Sci.,* 1949, v52, 292.

5. Hillman, R. W., Rosner, M. C., *J. Nutrition,* 1958, v64, 605.

6. Ferguson, M. W., Bridgforth, E., Quaife, M. L., Martin, M. P., Cannon, R. O., McGanity, W. J., Newbill, J., Darby, W. J., *ibid.,* 1955, v55, 305.

7. Rindi, G., Perri, V., *Intern. Z. Vitaminforsch.,* 1958, v28, 274.

8. Wechsler, I. S., Mayer, G. G., Sobotka, H., PROC. SOC. EXP. BIOL. AND MED., 1941, v47, 152.

9. Chieffi, M., Kirk, J. E., *J. Gerontol.,* 1951, v6, 17.

10. Kramer, M., *Intern. Z. Vitaminforsch.,* 1955-6, v26, 58.

11. Van Bruggen, J. T., Straumfjord, J. V., *J. Lab. & Clin. Med.,* 1948, v33, 67.

12. Harris, P. L., Hickman, K. C. D., Jensen, J. L., Spies, T. D., *Am. J. Public Health,* 1946, v36, 155.

13. Scrimshaw, N. S., Greer, R. B., Goodland, R. L., *Ann. N. Y. Acad. Sci.,* 1949, v52, 312.

14. Leitner, Z. A., Moore, T., Sharman, I. M., *Brit. J. Nutrition,* 1960, v14, 281.

15. Darby, W. J., Cherrington, M. E., Ruffin, J. M., PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 310.

16. Urbach, C., Hickman, K. C. D., Harris, P. L., *Exp. Med. Surg.,* 1952, v10, 7.

17. Lemley, J. M., Gale, R. G., Furman, R. H., Cherrington, M. E., Darby, W. J., Meneely, G. R., *Am. Heart J.,* 1949, v37, 1029.

18. Harris, P. L., Quaife, M. L., *J. Biol. Chem.,* 1944, v156, 499.

19. Postel, S., *J. Clin. Invest.,* 1956, v35, 1345.

20. Klatskin, G., Krehl, W. A., *ibid.,* 1950, v29, 1528.

21. Edwin, E. E., Diplock, A. T., Bunyan, J., Green, J., *Biochem. J.,* 1960, v75, 450.

22. Rose, C. S., Gyorgy, P., *Blood,* 1950, v5, 1062.

23. Mackenzie, J. B., *Pediatrics,* 1954, v13, 346.

24. Gordon, H. H., Nitowsky, H. M., Tildon, B. S., Levin, S., *ibid.,* 1958, v21, 673.

25. Gordon, H. H., Nitowsky, H. M., Cornblath, M., *A.M.A. Am. J. Diseases Child.,* 1955, v90, 669.

Received March 16, 1961. P.S.E.B.M., 1961, v107.

Phospholipid Analysis of Cellular Fractions of the Liver Following Administration of Single Dose of Choline.* (26632)

W. E. CORNATZER AND DUANE G. GALLO†

Guy and Bertha Ireland Research Laboratory, Department of Biochemistry, University of North Dakota School of Medicine, Grand Forks

Liver mitochondria, nuclei and microsomes are composed of 16-32% of phospholipids on a dry weight basis(1,2). These intracellular units contain enzymes which can oxidize fatty acids and other metabolites(3). Choline-containing phospholipids (lecithins) are probably an integral part of an enzyme system necessary for oxidization of fats and fatty acids since this is only phospholipid, except phosphatidylinositol which is lipotropic. The lipotropic effect of lecithins appears to act on the metabolism of fatty acids in the liver, rather than enhancing their mobilization in the form of plasma phospholipids(4). There is a progressive decrease in concentration in the whole liver of the lecithins and a diminished ability of the liver to synthesize these phospholipids in the dog(5) or rat(6) maintained on a choline-deficient diet. Diets low in protein reduce the level of total liver phospholipids(7,8). Administration of a single dose of choline stimulated lipid phosphorylation as shown by the increase of radioactive uptake of P^{32}(9,10,11) which occurs primarily in the lecithin fraction(12), involving mitochondria and nuclei(13). In view of the above findings, experiments were undertaken to determine the phospholipid analysis of various cellular fractions of liver following administration of a single dose of choline in an attempt to ascertain the role of these lipids in mitochondria and nuclei.

Methods. Male albino rats of the Wistar strain weighing 100-110 g were maintained on 5% casein-5% fat diet(10) supplemented with 1% guanidoacetic acid (a methyl group acceptor) for 2.5 weeks and divided into 2 groups. To one group various single doses of choline in 1 ml of water (0, 40, 75 and 150 mg) was administered by stomach tube and the animal sacrificed 6 hours later. A single

* Supported in part by grants from U. S. Atomic Energy Comm. and Nat. Inst. Health.

† Present address, Mead Johnson, Evansville, Ind.

dose of 150 mg of choline in 1 ml was administered to the other group and the animals sacrificed at 0, 3, 6, 10 and 18 hours later.

Three additional groups of rats were maintained on a dietary regime of 5% casein-5% fat(10), 25% casein-5% fat(14) and stock diet, Dakota Maid Fox Feed‡ (30% protein-5% fat) and sacrificed at the end of 4 weeks.

The livers were quickly removed, weighed on a Sartorius Balance. In most cases it was necessary to pool 2 livers for analyses. The liver was minced in an iced beaker and diluted 10-fold with cold 0.88 M sucrose. The mince was homogenized in a Potter-Elvehjem type tissue grinder with Teflon pestle for approximately 4 minutes in an ice bath, forced through a No. 20 gauge hypodermic needle to remove stroma, and centrifuged in a Servall Angle Centrifuge (Type RR-1), according to a modification of the procedures of Hogeboom *et al.*(15) and Griffiths and Pace(16) for separation of the cellular fractions by differential centrifugation. Mitochondria fraction: An aliquot of the homogenate was centrifuged at 1,000 × G for 15 minutes to remove nuclei. The supernatant was then centrifuged at 10,000 × G for 30 minutes and the mitochondria precipitate was washed twice with cold 0.15 M NaCl and finally recentrifuged at 10,000 × G for 20 minutes. The mitochondria fraction was then treated with 10% trichloroacetic acid (TCA) containing 0.4 M $MgCl_2$(17) to remove the acid-soluble phosphorus and was then recentrifuged at 10,000 × G for 10 minutes. The precipitate was washed with 5% TCA containing 0.4 M $MgCl_2$ and recentrifuged at 10,000 × G for 15 minutes. The mitochondria precipitate was then covered with ethanol for dehydration for 6 hours and then washed quantitatively into Soxhlet extracting thimbles with ethanol. Nuclear fraction: An aliquot of the homogenate was recentrifuged at 10,000 × G for 30 minutes. The precipitate was washed with cold 0.15 M NaCl and 10% and 5% TCA solutions and recentrifuged. The precipitate represents nuclei and mitochondria. The value of the nuclei component was obtained by difference. Homogenate fraction: The homogenate fraction was precipitated by addition of 10% TCA, centrifuged at 1,000 × G for 15 minutes, washed with 5% TCA and recentrifuged. The lipids from the cellular fractions were extracted with 95% ethanol for 6 hours in Soxhlet continuous extractors. The lipids were purified with chloroform(18). Lipid P(19), lecithin and cephalin(20) were determined on aliquots of the chloroform solution. Histological examination showed that the mitochondria and nuclear fractions obtained corresponded in appearance and staining properties with those described by Claude (21) and Hogeboom(15).

‡ North Dakota State Mill and Elevator Co., Grand Forks.

Results. Table I shows the effect of dietary protein level on total lipid P, lecithin P and cephalin of mitochondria, nuclei and homogenates. To evaluate the significance of results, the *t* test of significance(22) was applied to the difference between mean of controls and experimental values. The data show that a reduction of protein concentration in the diet produces a statistically significant decrease in total lipid P, lecithin P and cephalin P in liver mitochondria. There is a statistically significant decrease in lecithin P of nuclei and homogenates of liver of the animals fed the 5% casein diet as compared to the 30% protein. These data indicate that the lipid P concentration in mitochondria is directly related to dietary protein intake. This observation is in agreement with the studies carried out on feeding various proteins and analyzing total lipid phosphorus; the quantity and quality of dietary protein determines phospholipid content of the liver cell(7,8,23).

The effect of a single dose of choline at various time intervals on the phospholipids of mitochondria, nuclei and homogenates in choline deficient animals is shown in Table II. It is apparent that administration of a single dose of choline by stomach tube increases total lipid P and lecithin P concentration in the liver in 3 hours. There is a statistically significant increase in total lipid P and lecithin P at 3, 6, 10 and 18 hours in the mitochondria following administration of a single dose of choline when the animals

TABLE I. Effect of Dietary Protein Level on Phospholipids of Mitochondria, Nuclei and Homogenates.

		mg of P/g of wet tissue								
		Mitochondria			Nuclei			Homogenates		
Dietary protein, %	No. of rats	Total lipid P	Lecithin P	Cephalin P	Total lipid P	Lecithin P	Cephalin P	Total lipid P	Lecithin P	Cephalin P
30	20 (8)	.29 ± .06	.12 ± .03	.16 ± .03	.27 ± .10	.14 ± .04	.12 ± .06	1.07 ± .09	.54 ± .07	.49 ± .09
25	25 (9)	.17 ± .03*	.07 ± .02*	.09 ± .02*	.43 ± .05*	.21 ± .03*	.20 ± .03*	1.03 ± .02	.54 ± .06	.42 ± .03
5	31 (11)	.16 ± .07*	.05 ± .03*	.10 ± .03*	.22 ± .06	.09 ± .03†	.11 ± .03	.65 ± .05*	.27 ± .04*	.34 ± .04

Values in parentheses indicate No. of experiments. Figures preceded by ± sign indicate stand. dev. Test of significance was applied to difference between mean of control (stock diet) and experimental values. Probability for chance occurrence of this difference was: * <.01; † <.02.

TABLE II. Effect of Single Dose of Choline (150 mg) on Phospholipids of Mitochondria, Nuclei and Homogenates at Various Time Intervals.

		mg of P/g of wet tissue								
		Mitochondria			Nuclei			Homogenates		
Time, hr	No. of rats	Total lipid P	Lecithin P	Cephalin P	Total lipid P	Lecithin P	Cephalin P	Total lipid P	Lecithin P	Cephalin P
0	34 (10)	.17 ± .03	.04 ± .02	.13 ± .03	.13 ± .03	.05 ± .02	.06 ± .02	.55 ± .07	.14 ± .06	.37 ± .09
3	8 (8)	.22 ± .03†	.09 ± .01*	.12 ± .03	.14 ± .04	.06 ± .02	.06 ± .02	.71 ± .10*	.33 ± .04*	.34 ± .07
6	26 (13)	.26 ± .04*	.11 ± .02*	.14 ± .03	.11 ± .04	.05 ± .02	.05 ± .02	.75 ± .13*	.30 ± .05*	.38 ± .08
10	16 (12)	.22 ± .03*	.12 ± .02*	.10 ± .03‡	.19 ± .07‡	.08 ± .03†	.09 ± .04	.81 ± .17*	.37 ± .08*	.38 ± .09
18	8 (6)	.26 ± .02*	.09 ± .01*	.15 ± .03	.12 ± .02	.04 ± .01	.07 ± .01	.83 ± .03*	.32 ± .04†	.40 ± .05

Values in parentheses indicate No. of experiments. Figures preceded by ± sign indicate stand. dev. Test of significance was applied to difference between mean of controls and experimental values. Probability for chance occurrence of this difference was: * <.01; † <.02; ‡ <.05.

TABLE III. Effect of Single Dose of Choline on Phospholipids of Mitochondria, Nuclei and Homogenates in 6 Hours.

		mg of P/g of wet tissue								
		Mitochondria			Nuclei			Homogenates		
Choline admin., mg	No. of rats	Total lipid P	Lecithin P	Cephalin P	Total lipid P	Lecithin P	Cephalin P	Total lipid P	Lecithin P	Cephalin P
0	34 (10)	.17 ± .03	.04 ± .02	.13 ± .03	.13 ± .03	.05 ± .02	.06 ± .02	.55 ± .07	.14 ± .06	.37 ± .09
40	16 (10)	.21 ± .04†	.10 ± .02*	.11 ± .02	.15 ± .04	.06 ± .02	.08 ± .03	.73 ± .28	.27 ± .10*	.35 ± .15
75	8 (6)	.24 ± .02*	.12 ± .0 *	.11 ± .02	.13 ± .03	.06 ± .01	.06 ± .01	.53 ± .08	.21 ± .02‡	.22 ± .06†
150	26 (13)	.26 ± .04*	.11 ± .02*	.14 ± .03	.11 ± .04	.05 ± .02	.05 ± .02	.75 ± .13*	.30 ± .05*	.38 ± .08

Values in parentheses indicate No. of experiments. Figures preceded by ± sign indicate stand. dev. Test of significance was applied to difference between mean of controls and experimental values. Probability for chance occurrence of this difference was: * <.01; † <.02; ‡ <.05.

have been made choline deficient by administration of a methyl group acceptor, 1% guanidoacetic acid in the diet.

The effect of smaller doses of choline on total lipid P, lecithin P and cephalin P in mitochondria, nuclei and homogenates is shown in Table III. It is apparent that administration of single dose of 40 mg of choline in 6 hours produces a statistically significant increase in concentration of total lipid P and lecithin P in liver mitochondria. This increase of lecithin P concentration in the mitochondria following administration of single dose of choline gives further proof that the composition of these cellular units is affected by dietary intake. Recently Hartroft (24) has shown that liver mitochondria have an oblong or oval profile in electronmicrographs but their shapes become rounded in profile in early choline deficiency. In later stages of choline deficiency a striking increase in size of these mitochondria was observed.

Summary. 1. The effect of a single dose of choline on total lipid phosphorus, lecithin P, and cephalin P of mitochondria, nuclei and homogenates of the liver was studied in choline deficient rats. 2. Administration of single dose of choline 40, 75 and 150 mg significantly increased in 6 hours the total lipid P, and lecithin P concentration of liver mitochondria. 3. A statistically significant increase in total lipid P and lecithin P of liver mitochondria and homogenates occurred in 3, 6, 10 and 18 hours following administration of a single dose of choline. 4. Diets low in protein reduce the level of total lipid P, lecithin P and cephalin P in liver mitochondria.

1. Swanson, M. A., Artom, C., *J. Biol. Chem.*, 1950, v187, 281.

2. Spiro, M. J., McKibbin, J. M., *ibid.*, 1956, v219, 643.

3. Kennedy, E. P., Lehninger, A. L., *ibid.*, 1949, v179, 957.

4. Artom, C., *Nature*, 1953, v171, 347; *J. Biol. Chem.*, 1953, v205, 101.

5. Diluzio, N. R., Zilversmith, D. B., *ibid.*, 1953, v205, 867.

6. Artom, C., *Liver Injury*, Editor, F. A. Hoffbauer, Josiah Macy, Jr. Foundation, New York, 1951; Artom, C., Cornatzer, W. E., *J. Elisha Mitchell Sci. Soc.*, 1949, v65, 191.

7. Rosenfeld, B., Lang, J. M., *Canadian J. Biochem. and Phy.*, 1957, v35, 845.

8. Kosterlitz, H. W., *J. Physiol.*, 1947, v106, 194.

9. Perlman, I., Chaikoff, I. L., *J. Biol. Chem.*, 1939, v127, 211.

10. Artom, C., Cornatzer, W. E., *ibid.*, 1947, v171, 779.

11. Snyder, F., Cornatzer, W. E., Simonson, G. E., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 670.

12. Artom, C., Cornatzer, W. E., *J. Biol. Chem.*, 1948, v176, 949.

13.. Cornatzer, W. E., Snyder, F., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 363.

14. Cornatzer, W. E., Harrell, G. T., Jr., Cayer, D., Artom, C., *ibid.*, 1950, v73, 492.

15. Hogeboom, G. H., Schneider, W. C., Palade, G. E., *J. Biol. Chem.*, 1948, v172, 619; Schneider, W. C., Hogeboom, G. H., *ibid.*, 1950, v183, 123.

16. Griffiths, M., Pace, N., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 771.

17. Johnson, R. M., Dutch, P. H., *ibid.*, 1951, v78, 662.

18. Artom, C., *J. Biol. Chem.*, 1941, v139, 1953.

19. Fiske, C. H., Subbarow, Y., *ibid.*, 1925, v66, 375.

20. Hack, M. H., *ibid.*, 1947, v169, 137.

21. Claude, A., *J. Exp. Med.*, 1946, v84, 51.

22. Chambers, E. G., *Statistical Calculation for Beginners*, N. Y. Cambridge Univ. Press, 1952, 2nd edition.

23. Fishman, W. H., Artom, C., *J. Biol. Chem.*, 1946, v164, 307.

24. Hartroft, W. Stanley, *Proc. Fifth Int. Cong. Nutrition, Panel III Lipides in Health and Disease*, Washington, D.C., 1960, 21.

Received March 16, 1961. P.S.E.B.M., 1961, v107.

Immediate Decrease in Respiratory $C^{14}O_2$ Excretion Following Simultaneous Intravenous Administration of $NaHC^{14}O_3$ and Acetazoleamide in the Rat. (26633)

JAMES L. SPRATT** AND GEORGE V. LEROY (Introduced by L. O. Jacobson)
Argonne Cancer Research Hospital and Depts. of Pharmacology and Medicine, University of Chicago, Chicago, Ill.*

Speculating on the metabolic sequelae of carbonic anhydrase inhibition, Berliner and Orloff(1) concluded that the effects of carbonic anhydrase inhibitors on CO_2 elimination would be an immediate, though transient, decrease in CO_2 output and the appearance of a difference between alveolar CO_2 tension and that found in arterial blood. Since that time different authors have reported variable effects of the potent carbonic anhydrase inhibitor, acetazoleamide, on output of respiratory CO_2(2-9). The consensus of these various studies is that although acetazoleamide causes an initial hyperventilation that may produce an increased CO_2 output of brief duration, CO_2 output is usually transiently depressed due to inhibition of erythrocyte carbonic anhydrase. The investigation reported here concerns the influence of acetazoleamide on excretion of $C^{14}O_2$ derived from C^{14}-labeled bicarbonate using a device which continuously measures the radioactivity of expired air. Our method allows direct demonstration of Berliner and Orloff's hypothesis in the intact animal, without necessitating recourse to indirect estimates of CO_2 movement based on application of acid-base equations.

Methods. Female Sprague-Dawley rats weighing 65-85 g were starved overnight prior to experiment. Four experimental animals were injected intravenously with 0.1 μc of $NaHC^{14}O_3$ (2.39 mC/mmol in 0.1 cc), followed immediately by acetazoleamide-sodium†, 70 mg/kg (in 0.5 cc/100 g body weight). Nearly-simultaneous injection of $NaHC^{14}O_3$ and acetazoleamide-Na (or saline) was accomplished by means of a double syringe with a 3-way stopcock. This procedure allowed essentially simultaneous administration of the two materials with no variation in $NaHC^{14}O_3$ dosage. Four control animals were injected with $NaHC^{14}O_3$ and saline in the same manner.

Similar animals were pretreated with intraperitoneal acetazoleamide (20 mg/kg) at 30 minutes prior to administration of intraperitoneal $NaHC^{14}O_3$ (5 μc/kg) and their production of respiratory $C^{14}O_2$ likewise studied.

Immediately after injection of the radiotracer each animal was placed in a sealed container attached to a continuous $C^{14}O_2$ monitor. The apparatus, described in detail elsewhere(10), supplies the animal chamber with a constant flow of CO_2-free air. The outflow of the chamber passes through a 4 π G-M counter‡ connected to a scaler§, a rate meter‖ and a graphic recorder¶. The graphic data are correlated with the total counts accumulated on the scaler. After corrections for background and counter efficiency, the per cent of dose recovered per unit of time can be calculated.

Results. The results are presented in Fig. 1 and Table I. When $NaHC^{14}O_3$ and acetazoleamide (70 mg/kg) were given nearly simultaneously *i.v.*, there was an obvious disparity between the curves for treated and control animals (Fig. 1). The difference be-

* Operated by University of Chicago for U. S. Atomic Energy Commission.

** Present address: Dr. James L. Spratt, Department of Pharmacology, State University of Iowa, Iowa City, Iowa.

† Supplied as the sodium salt (Diamox), by the Lederle Laboratories Division, American Cyanamid Co.

‡ Model 4P5, Instrument and Development Products Co., Chicago, Illinois.

§ Model 162A, Atomic Instrument Co., Cambridge, Massachusetts.

‖ Model 1620A, Nuclear Chicago Corp., Chicago, Illinois.

¶ Recti-riter, Texas Instruments, Inc. Houston, Texas.

TABLE I. Cumulated Per Cent of Injected Dose. Data expressed as mean ± mean deviation.*

Time after $NaHC^{14}O_3$ inj., min.	Intraper. $NaHC^{14}O_3$		Intrav. $NaHC^{14}O_3$	
	Saline control (3)	Acetazoleamide, 20 mg/kg I.P. (4)	Saline control (4)	Acetazoleamide, 70 mg/kg I.V. (4)
5	6.12 ± 1.32	6.80 ± 1.48	13.78 ± 1.80	7.69 ± 0.50
10	21.61 ± 3.46	20.50 ± 0.68	30.92 ± 3.74	23.49 ± 1.48
15	32.04 ± 5.19	31.56 ± 1.95	39.57 ± 5.10	34.46 ± 2.70
30	45.06 ± 7.39	46.96 ± 5.79	50.46 ± 5.95	51.21 ± 5.05

* No. of animals indicated by parentheses.

tween the rate curves at 5 minutes was highly significant ($p<0.01$). Similarly, the difference between the cumulated curves at 10 minutes was significant at $p<0.05$. By 15 minutes the marked changes had subsided.

Discussion. These experiments indicate that the effect of a potent carbonic anhydrase inhibitor on excretion of carbon dioxide can be demonstrated when such an inhibitor, acetazoleamide, and bicarbonate are injected simultaneously intravenously. When the drug is administered 30 minutes prior to injection of the source of tracer $C^{14}O_2$, the inhibitory effect on expired CO_2, although evident, is not significant. The inconclusive result may be due to the large variance of the experimental values (Table I); or may be the consequence of establishment of a new equilibrium among the different forms of carbon dioxide in blood and tissues. The discrepancies between the 30-minute pretreated and the simultaneously treated acetazoleamide animals are consistent with Mithoefer's observations in the dog(8), that the primary effect of acetazoleamide on respiratory CO_2 excretion is manifest very quickly and subsides in 20 minutes.

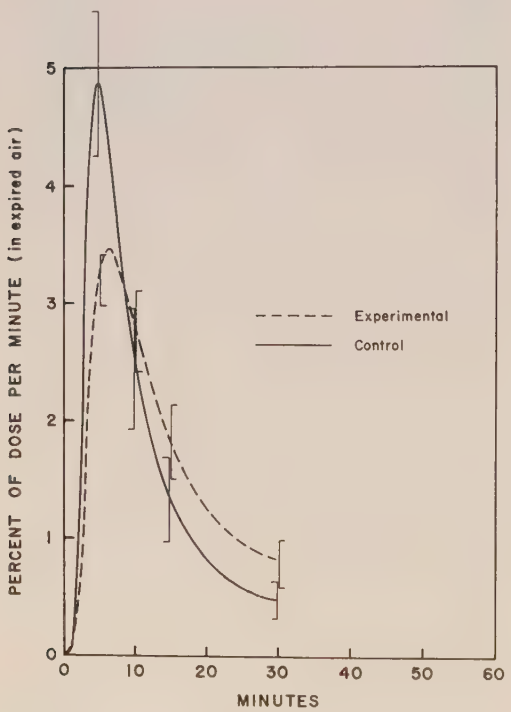


FIG. 1. Rate of excretion of $C^{14}O_2$ in expired air following intravenous injection of $NaHC^{14}O_3$. Experimental animals (broken line) were administered acetazoleamide, 70 mg/kg, simultaneously with bicarbonate. Points on smoothed curves represent means ± standard deviations.

Summary. The effect of acetazoleamide on rate of expiration of carbon dioxide was studied in rats using carbon-14 labeled bicarbonate. A marked transient reduction in expired $C^{14}O_2$ was observed during the first 10 minutes after simultaneous intravenous injection of the drug (70 mg/kg) and $NaHC^{14}O_3$. The rate of appearance of $C^{14}O_2$ was reduced 35 ± 7% in the treated animals 5 minutes after injection. When the animals were pretreated (30 minutes) with acetazoleamide (20 mg/kg) and when the labeled $NaHC^{14}O_3$ entered the blood slowly as a result of intraperitoneal injection, the reduction in rate of $C^{14}O_2$ elimination was not nearly as marked.

The authors wish to acknowledge the technical assistance of Mr. Gar Bo Ho and Mrs. Maija Mednieks.

1. Berliner, R. W., Orloff, J., *Pharmacol. Rev.*, 1956, v8, 137.

2. Cain, S. M., Leeds, R. F., Travis, L. P., Otis, A. B., *Fed. Proc.*, 1958, v17, 22.

3. Carter, E. T., Clark, R. T., Jr., *J. Appl. Physiol.*, 1958, v13, 42.

4. Pocidalo, J. J., Lissac, J., Amiel, J. L., Blayo, M. C., *Rev. fr. clin. Biol.*, 1958, v3, 1079.

5. Mithoefer, J. C., Davis, J. S., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 797.

6. Amiel, J. L., Pocidalo, J. J., Lissac, J., Blayo, M., *Rev. fr. clin. Biol.*, 1959, v4, 44.

7. Lissac, J., Amiel, J. L., Cophignon, J., Pocidalo, J. J., *ibid.*, 1959, v4, 157.

8. Mithoefer, J. C., *J. Appl. Physiol.*, 1959, v14, 109.

9. Chinard, F. P., Enns, T., Nolan, M. F., *Am. J. Physiol.*, 1960, v198, 78.

10. Spratt, J. L., LeRoy, G. V., in preparation.

Received March 20, 1961. P.S.E.B.M., 1961, v107.

Factors Influencing Experimental Modification of Ehrlich Ascites Tumor Cells.* (26634)

JOHN PAUL KALTENBACH AND W. B. LYONS (Introduced by R. B. Jennings)
Dept. of Pathology, Northwestern University Medical School, Chicago, Ill.

Previous studies have shown that the dye nigrosin can be used to differentiate unmodified cells, whose membrane is intact (unstained cells), from modified cells whose membrane permeability has been altered experimentally or by death of the cell (stained cells)(1). This differential staining technic has been used to quantitate metabolic data of ascites tumor cells and suspensions of cells obtained from solid tumor as to the proportion of activity due to stained or unstained cells(2). Recently, a modification procedure was described to increase experimentally the permeability of ascites tumor cells by brief exposure of the cells to a hypotonic medium (1). It is of considerable interest that the modification procedure does not result in uniform modification of all cells present, *i.e.*, some cells are apparently resistant to such injury-provoking conditions as hypotonic shock. The following experiments were done to determine whether or not any of the variables involved in experimentally increasing the permeability of Ehrlich ascites tumor cells would influence the resistance and/or lack of resistance of these cells to these experimental conditions.

Material and methods. Preparation of ascites tumor cells. A hypotetraploid subline of Ehrlich ascites tumor cells (EAT cells), carired in Strong A mice, was used in all experiments. The ascites tumor from each animal was collected in 15 ml centrifuge tubes containing 2 to 4 ml of cold buffered saline (9 parts of 0.9% NaCl + 1 part M/15 phosphate buffer). Cold saline was used because it partially prevents coagulation of the ascites fluid. The cells were centrifuged from the ascitic fluid, and twice washed in buffered saline. Centrifugation was carried out at about 125 to 150 × gravity for 3 minutes, then increased to about 200 × gravity for an additional 2 to 3 minutes. This centrifugation procedure aided in separating the tumor cells from the red blood cells and white cells; the latter tended to remain in the supernatant fluid. Tumors that were grossly contaminated with erythrocytes were discarded.

Preparation of modified tumor cells (MA cells). The ascites tumor cells were modified according to the following procedure: 0.5 ml of cells (about 20 × 10^6 cells) in buffered saline were diluted with distilled water to 4.0 ml. The cells were allowed to stand at room temperature for 4 minutes, then 4.0 ml of double strength saline (1.8%) were added to stop the modification. This procedure was altered only in those experiments described in the text that were designed to test the effect of different salt concentrations on the modification procedure.

Staining procedure: Cells were diluted with 0.2% water-soluble nigrosin dissolved in buffered saline. Differential cell counts of stained and unstained cells were made in

* This investigation was supported by PHS research grant, Nat. Cancer Inst., U.S.P.H.S.

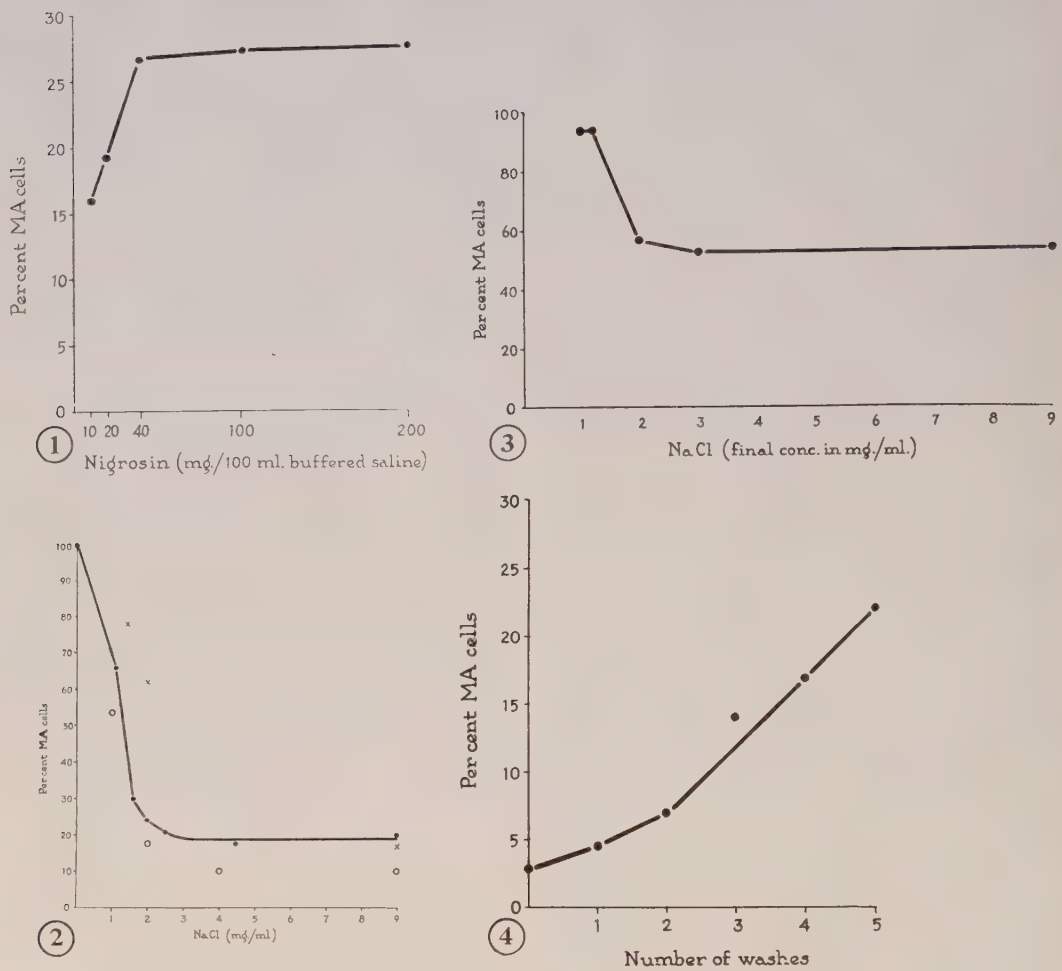


FIG. 1. Relationship between nigrosin concentration and number of cells stained.
FIG. 2. Resistance of EAT cells to low concentrations of NaCl (solid circles), buffered NaCl (open circles), and KCl (crosses).
FIG. 3. Concentration of NaCl required to stop modification of EAT cells.
FIG. 4. Relationship between number of times EAT cells are washed (centrifuged and resuspended) and increase in modified cells.

a hemocytometer according to the procedure described previously(1). Results are presented as percent modified cells (percent MA cells), as total cell count did not change appreciably during these experiments. Cell counts were done in duplicate and the average recorded.

Results and discussion. Effect of nigrosin concentration. Various concentrations of nigrosin were tested to determine the range of optimum dye concentration that would give reproducible results for quantitative staining of the modified tumor cells. Dye concentrations of 0.2% and less were used because previous work has shown that 0.2% nigrosin had no deleterious effect on cell membrane permeability or cell metabolism(1).

EAT cells (20×10^6 cells) were modified as described in Methods. Differential cell counts were made using concentrations of nigrosin from 0.01 to 0.2%. Fig. 1 shows that the number of stained cells increases with increasing concentrations of nigrosin, and that at least 0.1% nigrosin is required to obtain maximum and consistent stained-cell counts.

The importance of determining optimum dye concentration for quantitative staining of

modified EAT cells is shown by the above results. Hanks and Wallace(3) have also indicated the importance of determining optimum dye concentration in studies using the more fragile tissue culture cells. Dye inclusion in these cells apparently suggests loss of viability.

Effect of NaCl concentration. The purpose of these experiments was to determine the lowest concentration of NaCl required to prevent modification of EAT cells. The effect of buffered saline, KCl, and cell concentration was also investigated.

The EAT cells were modified as described in Methods, except that the cells were diluted with various concentrations of NaCl, buffered NaCl, or KCl instead of distilled water. Fig. 2 shows that the number of cells modified did not change appreciably until NaCl concentration was decreased to less than 3 mg/ml. Cell lysis occurred following exposure to solutions containing less than 1.0 mg NaCl/ml. Similar results were obtained when buffered saline was substituted for NaCl. Substitution of KCl in the modification procedure resulted in a somewhat greater number of modified cells. The results are similar whether based on absolute or equivalent ionic concentration. Varying the total number of cells in the aliquot to be modified from 10^7 to 40^7 did not affect the results.

These findings show that the final concentration of sodium chloride in the cell suspension is a primary factor influencing modification of EAT cells under the conditions employed, *viz.*, brief hypotonic shock. Moreover, the abrupt increase in number of modified cells at low concentrations of sodium chloride lends to the difficulty of standardizing the modification procedure. Also indicated is the apparent stability of the semipermeable character of the cell membrane of EAT cells over a relatively large range of sodium chloride concentrations. It is noteworthy that certain other cells, in contrast to EAT cells, are apparently adversely affected by even 0.85% (normal) saline, *e.g.*, tissue culture cells(4). The results obtained with EAT cells may be due, in part, to the relative impermeability of the tumor cells to sodium chloride(5).

Concentration of NaCl to stop modification. In the previous experiment, cells were modified in various concentrations of sodium chloride. In this experiment, the cells were modified in distilled water and various concentrations of sodium chloride were added to stop the modification of the cells. The purpose was to establish the fact that a certain minimal concentration of sodium chloride is required to prevent modification. Positive results would confirm the findings of the previous experiment.

Cells were modified as described in Methods, except that various concentrations of sodium chloride were used to stop the modification of cells, in place of double strength saline. Fig. 3 shows that a concentration of 3 mg/ml of sodium chloride was sufficient to stop the modification of the cells. This corresponds to the minimum concentration of sodium chloride that prevented modification in the previous experiment. These results also indicate that gross changes in the semipermeable nature of the EAT cell are manifested at, or below, a concentration of 3 mg/ml sodium chloride.

The ability of salt solutions to prevent modification of cells has also been shown to occur with plant cells. Thin slices of red beet root (*Beta vulgaris*) subjected to distilled water continued to leak red pigment for several hours, but the leakage stopped as soon as the slices were transferred to 0.1 to 0.2 molar sodium or potassium chloride solution(6).

Effect of time on modification. As would be expected, more and more cells are modified the longer the cells are kept in water during the modification procedure. However, the relationship between time and percent of modified cells is not linear, apparently because the amount of protein (ascitic fluid) and salts contained in the aliquot of cells to be modified tempers the number of cells that become modified in any period of time. Thus, it is necessary to wash the cells to obviate the variable effects of ascitic fluid carried with the cells.

Effect of washing the cells. Manipulation of the ascites tumor cells may also cause a change in cell membrane permeability. This

is probably due to the elution of low molecular weight cofactors and substrates from the cells so that the cells can no longer maintain the integrity of their semi-permeable membrane. Le Page(7) has shown that 5 washes (centrifuging and resuspending the cells) of EAT cells will deplete all or almost all of the intracellular free glycine. Fig. 4 shows that repeated washings of EAT cells in buffered saline result in an increasing number of cells which take up the nigrosin dye indicating a change in membrane permeability. The effect of time on modification of the cells and the effect of repeated washing on the cells remain empirical factors in the modification procedure, since both are influenced by the amount of protein and salts present in the aliquot to be modified. Studies are in progress to attempt control of these factors through use of a relatively inert suspending medium.

Of particular interest with regard to previous studies and to the results herein obtained is the fact that all cells in a given population were not similarly nor simultaneously affected by the experimental conditions employed. Inspection of the figures shows that only a certain percentage of the cells were affected as measured by dye (nigrosin) uptake, although all cells were simultaneously subjected to the same environment. The change in membrane permeability or the reaction of these cells to a particular environment appears to be dependent upon the cell *per se* and seems to vary from cell to cell. It is not possible at this time to definitely ascribe this phenomenon to any particular biochemical or physiological characteristic or function of the cell.

Summary. A study was made of factors involved in modifying the semi-permeable nature of the cell membrane of Ehrlich ascites tumor cells. It was found that a minimum concentration of 0.1% nigrosin was necessary for accurate differential quantitation of modified and unmodified tumor cells. The cells were not visibly affected by hypotonic saline solutions as low as 3.0 mg NaCl/ml. Lower concentrations resulted in changes in membrane permeability, and lysis occurred at concentrations of 1.0 mg/ml, or less. Not all cells of a given population were affected similarly by experimental hypotonic shock.

1. Kaltenbach, J. P., Kaltenbach, M. H., Lyons, W. B., *Exp. Cell Research,* 1958, v15, 112.

2. Kaltenbach, J. P., Kaltenbach, M. H., *Ann. N. Y. Acad. Sci.,* 1956, v63, 977.

3. Hanks, J. H., Wallace, J. H., Proc. Soc. Exp. Biol. and Med., 1958, v98, 188.

4. Pomerat, C. M., Overman, R. R., *Z. f. Zellforsch.,* 1956, v45, 2.

5. Lucke, B., Parpart, A. K., *Cancer Research,* 1954, v14, 75.

6. Ginzburg, B. Z., *Nature,* 1959, v184, 1073.

7. Le Page, G. A., *Cancer Research,* 1953, v13, 178.

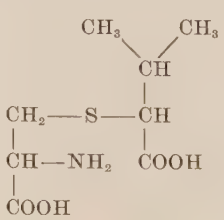
Received March 30, 1961. P.S.E.B.M., 1961, v107.

A Convenient Preparation of Isovalthine.* (26635)

Lemuel D. Wright

Graduate School of Nutrition, Cornell University, Ithaca, N. Y.

Mizuhara *et al.* and Oomori and Mizuhara have recently described the discovery(1,2), isolation(1,2), identification(3), and synthesis(3) of a new amino acid, S-(isopropyl-carboxymethyl)-cysteine, to which they gave the name isovalthine. This amino acid was first observed in the urine of hypercholesterolemic individuals.



* Supported by research grants from Nat. Science Foundation and Nat. Inst. Health, and by funds made available through State Univ. of New York.

Studies on the biogenesis of the compound in this laboratory were hampered by the inconvenience of using liquid ammonia required in the original synthesis. This communication describes an easier synthesis and details of an isolation procedure whereby the compound is easily made available in quantity.

Methods and materials. Synthesis and isolation of isovalthine. Five g of cysteine (0.041 M, free base, Nutritional Biochemicals Corp.) are dissolved in 100 ml of water. Eighteen g of α-bromoisovaleric acid (0.1 M, Eastman-Kodak Co., some other preparations have shown the presence of significant amounts of extraneous material) are added followed by 7.3 g solid sodium hydroxide (0.18 M, pellets) which are added with swirling over a period of a few minutes. The mixture is allowed to stand at room temperature for 2 hours and is then heated on a steam bath for one hour. The amount of α-bromoisovaleric acid used to react with 5 g cysteine (0.041 M) has been varied from 9 (0.05 M) to 18 (0.1 M) g and the time of heating varied from a minimum of 30 min of heating directly after mixing to a maximum of one hour of heating after standing at room temperature for 2 hours. The amount of α-bromoisovaleric acid used or the amount of heating employed, at least within the limits mentioned, does not influence the yield or identity of the final product obtained. In all instances where the quantities of reactants have been varied the sodium hydroxide has been varied correspondingly to maintain an amount equivalent to the carboxyl groups of cysteine and of α-bromoisovaleric acid plus the hydrobromic acid eliminated if all the cysteine used reacted with α-bromoisovaleric acid.

The reaction mixture is poured onto a column of 150 ml of Dowex-1, 50-100 mesh, freshly prepared in the OH^- form by pretreatment in the column with several hundred ml of 10-20% potassium hydroxide and then with water. The reaction mixture is allowed to run through the column at a rate of about 10 ml/min and is followed with several hundred ml of water. The isovalthine, now free of cations, together with excess α-bromoisovaleric acid is eluted from the column with 0.1 N hydrochloric acid. 100 ml fractions of the run-through and eluates are collected. 0.01 ml amounts of the various fractions are spotted on a sheet of Whatman #1 paper and developed by ascending chromatography in butanol:acetic acid:water (160:40:40). The isovalthine is located with ninhydrin (RF of about 0.53) and those fractions containing the isovalthine combined.

The isovalthine fractions from the Dowex-1 column are poured onto a column of 200 ml of Dowex-50, 50-100 mesh, freshly prepared in the H^+ form by pretreatment in the column with several hundred ml of 5 N hydrochloric acid and then with water. The isovalthine fraction is allowed to run through the column at a rate of about 10 ml/min and is followed with several hundred ml of water. The isovalthine, now free of excess α-bromoisovaleric acid, is eluted from the column with 2 N ammonia. 100 ml fractions of the run-through and eluates are collected. 0.01 ml amounts of the various fractions are spotted on a strip of paper. The isovalthine is located with ninhydrin and those fractions containing isovalthine combined and concentrated to a small volume in a rotary evaporator at a temperature not exceeding 70°C. A white precipitate, identified as cystine, sometimes appears on concentrating the Dowex-50 eluates. This should be removed before proceeding to the next step.

The isovalthine concentrate from the Dowex-50 column is then poured onto a column of 150 ml of Dowex-1, 50-100 mesh, freshly prepared in the Ac^- form by pretreatment in the column with several hundred ml of 5 N acetic acid and then with water. The concentrate is allowed to run through the column at a rate of about 10 ml per min and is followed with several hundred ml of water. The isovalthine from which essentially all of the contaminating cystine and/or cysteine has now been removed is eluted from the column with 2 N acetic acid. 100 ml fractions of the run-through and eluates are collected. 0.01 ml amounts of the various fractions are spotted on paper, developed in butanol:acetic acid:water and treated with ninhydrin. Those fractions containing the iso-

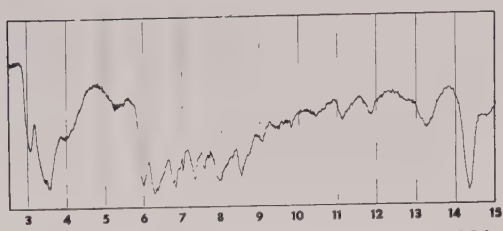


FIG. 1. Infrared absorption curve of isovalthine synthesized from L-cysteine and DL-α-bromoisovaleric acid. Figures on abscissa denote wavelengths in microns.

valthine are concentrated to a syrup *in vacuo* with the rotary evaporator and crystallized in a thin layer on the interior of the flask by constant motion of the flask until solidification of the product occurs. Excess acetic acid is removed with a stream of nitrogen.

The isovalthine is dissolved in a small volume of water and treated with norite. Ethanol to a concentration of about 50% is added to the hot filtrate. The mixture is cooled and crystallization occurs. The crystallization is apt to be slow and is facilitated in subsequent crystallizations by seeding with isovalthine. Several crops of crystals are obtained by repetition of the crystallization with successively smaller volumes. The yield of once-crystallized material obtained in several crops will amount to about 3-5 g or a yield of about 33-55%. An analysis of a typical batch of first crop material is as follows:

	C	H	N	S
Calculated for $C_8H_{15}NO_4S$	43.42	6.83	6.33	14.49
Found	42.81	6.83	6.16	14.25

Analysis by Scandinavian Microanalytical Laboratory

The product is obtained in the form of clusters of needles. It melts (with decomposition) at 185°. Isovalthine prepared as described is free of extraneous ninhydrin-reacting material. The infrared absorption curve of a typical preparation is given in Fig. 1.

Spacial configuration. From a consideration of the structure of isovalthine it is apparent that a synthesis involving L-cysteine and DL-α-bromoisovaleric acid as moieties should yield 2 diastereoisomers (L-cysteine-D-α-bromoisovaleric acid and L-cysteine-L-α-bromoisovaleric acid). Attempts at separating the diastereoisomers by fractional crystallization have been unsuccessful. Thus successive crops of crystals from a preparation have, within experimental error, the same optical rotation. Specific rotations of isovalthine as obtained by the procedures described when determined at 21-24° at concentrations of 10 mg/ml in a 4 dec tube with a white light and simulated D line filter are as follows:

Solvent	[α]
Water	− 7°
2 N Hydrochloric acid	+18°

RF values. Isovalthine prepared as described has RF values in the following systems as indicated; butanol:acetic acid:water (160:40:40), 0.53; butanol:ethanol:water (160:40:40), 0.12; butanol saturated with water, 0.04; pyridine:water (130:70), 0.35; buffered phenol(4), 0.28.

Summary. A convenient preparation of the new amino acid isovalthine is described. Some characteristics of the synthetic material are given.

1. Mizuhara, S., Okuda, K., Kurahashi, K., Iwado, Y., *J. Biochem.*, 1959, v46, 101.

2. Mizuhara, S., Oomori, S., *Arch. Biochem. and Biophys.*, 1961, v92, 53.

3. Oomori, S., Mizuhara, S., *Biochem. and Biophys. Res. Comm.*, 1960, v3, 343.

4. Berry, H. K., Sutton, H. E., Cain, L., Berry, J. S., *Univ. Texas Publ.*, 1951, #5109, 22.

Received April 6, 1961. P.S.E.B.M., 1961, v107.

Inhibition of Glucose Oxidation by 6-Deoxy-D-Glucose.*† (26636)

JAMES L. BROOKS, ELEANOR CHRISTENSEN, CHARLES J. STEWART, AND ARNE N. WICK
Chemistry Department, San Diego State College, San Diego, Calif.

Considerable evidence supports the concept for some kind of membrane transport system for intracellular transfer of sugars. However, there has not been any fundamental advance in understanding this transport mechanism. It is not known whether all sugars enter cells by one process or whether there are several processes, each of which affects only one sugar or a group of structurally related sugars. To gain further information on the cell permeation mechanism of sugars, we have carried out a series of experiments to determine if the sugar analog 6-deoxy-D-glucose (6-DOG) could act as an effective inhibitor of glucose metabolism in mammalian tissue. 6-Position substituted sugars, which cannot be phosphorylated by hexokinase, are considered unlikely to affect glucose metabolism in mammalian cells beyond the hexokinase step. Thus, the inhibition, if any, produced by 6-position modified glucose analogs should be less complex than that produced by analogs modified in, for example, the 2-position.

Methods. The effect of 6-DOG on glucose metabolism was measured by means of tracer technics in rat kidney cortex slices, rat diaphragm, and mouse adipose (epididymal) tissue. Glucose metabolism was followed by collection of the $C^{14}O_2$ produced by the tissues during incubation with glucose-U-C^{14} plus varying amounts of the test substance 6-DOG. With diaphragm, the tissue incorporation of C^{14} was also determined. In one experiment adipose tissue was incubated with 6-deoxy-D-glucose-U-C^{14} to determine if this substance was oxidized to $C^{14}O_2$.

Biological systems. Standardized procedures were used throughout. The incubation medium was Krebs-Ringer-phosphate buffer (calcium free). All incubations were at 37°C for 2 hours. Kidney and diaphragm tissues were from Sprague-Dawley rats. Kidney cortex was sliced with a Stadie-Riggs microtome; 150 mg of wet tissue was used per reaction flask. Paired rat hemi-diaphragms were incubated separately, with one serving as control for the other. Adipose tissue was from White Swiss mice. Of each pair of fat pads, of approximately 150 mg wet weight, one served as a control.

Preparation of 6-deoxy-D-glucose. Beta-1,2,3,4 - tetraacetyl - 6 - deoxy - D - glucose was prepared by a modification of the method of Hardegger and Montavon(1). This compound yielded 6-DOG on acid hydrolysis. The method was modified in 2 ways. In preparation of beta-1,2,3,4-tetraacetyl-6-iodo-D-glucose from beta-1,2,3,4-tetraacetyl-6-tosyl-D-glucose, the acetic anhydride solution of the product was flash evaporated, rather than being poured into water, and the solid resulting from flash evaporation was washed with water and recrystallized from 95% ethanol. The second modification was that beta-1,2,3,4-tetraacetyl-6-deoxy-D-glucose was prepared directly from the thiouronium iodide using methanol as the solvent rather than butanol mentioned in the original procedure. To do this, 30 g of the thiouronium iodide were dissolved in 550 ml of absolute methanol, 66 g of Raney nickel added, and the mixture stirred for 3 hours. The methanol was filtered and flash evaporated, and the residue crystallized from 1-butanol.

To prepare 6-DOG, 11 g of Amberlite 120 resin, type 3 (Rohm and Haas product), were washed 5 times with 5% sulphuric acid, then with distilled water until the rinse water was neutral. The resin was placed in 100 ml of water and 10 g of beta-1,2,3,4-tetraacetyl-6-deoxy-D-glucose were added. This was refluxed for 4.5 hours with stirring. The solution was filtered and lyophilized to dryness. The 6-DOG was crystallized from absolute ethanol. The M.P. was 146 to 147°C. One

* Supported by grants from U. S. Public Health Service to Arne N. Wick.

† Taken in part from M. S. Thesis of James L. Brooks, San Diego State College, 1961.

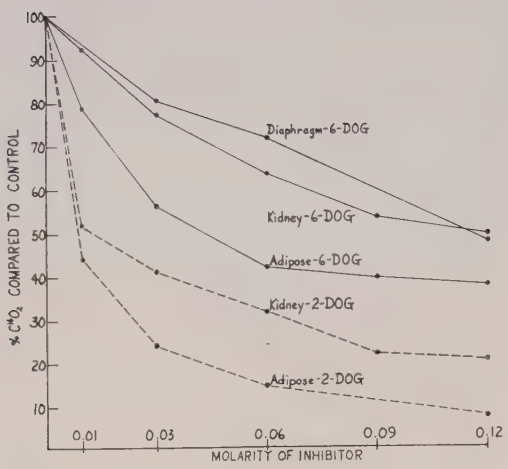


FIG. 1. Effect of 6-DOG and 2-DOG on oxidation of glucose-U-C^{14} by adipose, kidney, and diaphragm tissues.

batch was subjected to carbon and hydrogen analysis.‡ Found: 43.43% carbon and 7.53% hydrogen. Calculated for $C_6H_{12}O_5$: 43.90% carbon and 7.37% hydrogen. The osozone melted at 186°C as compared to the value 186 to 187°C given by Fischer and Zach(2). The labeled compound was prepared by starting was glucose-U-C^{14}.

Results. One experiment run with quadruplicate samples of adipose tissue (with and without insulin) and using 6-DOG-U-C^{14} as the sole substrate indicated that this compound is not oxidized to $C^{14}O_2$. No significant count above background was detected using the tagged 6-DOG at a concentration of 0.03M. A rate of 6-DOG oxidation 3% that of glucose would have given a counting rate twice normal background. The data are not included in this report.

Fig. 1§ shows the effect of 6-DOG on oxidation of glucose (0.01M). Experiments carried out simultaneously with 2-deoxy-D-glucose (2-DOG) are also shown for comparison purposes. It is apparent that 6-DOG inhibits glucose oxidation to a considerable degree, although the inhibition is not equivalent to that observed with equimolar 2-DOG. With the mammalian systems studied, 6-DOG inhibits oxidation of glucose 50 to 60% at a concentration ratio for 6-DOG to glucose of 12:1. Mouse adipose tissue is a much more sensitive tissue than etiher rat kidney slices or diaphragm tissue. With adipose tissue, a 6:1 ratio of 6-DOG to glucose inhibits glucose oxidation 55%.

To enhance the inhibiting effect of 6-DOG and to demonstrate the reversible nature of the inhibition, the effect of 0.06M 6-DOG on oxidation of glucose by mouse adipose tissue was examined at several different glucose concentrations. The data in Table I indicate that the inhibition produced by 6-DOG is reversible.

In Table II are results of 2 experiments concerning the effect of 6-DOG on glucose metabolism in rat diaphragm tissue. Besides showing that 6-DOG to glucose ratios of 6:1 and 12:1 inhibit glucose oxidation 26 and 52% respectively, the data show that the reduction in amount of C^{14} activity retained within this tissue was approximately proportional to the reduction in rate of glucose oxidation. The tissue incorporation of C^{14} was measured by technics involving wet combustion of the rinsed incubated tissue.

Discussion. 6-Deoxyglucose has been reported by Woodward *et al.*(3) to have no effect on glucose metabolism in yeast. In their studies the highest ratio of 6-DOG to glucose used was 2:1. The data presented here with mammalian tissue for higher ratios of analog to glucose show that this analog

TABLE I. Competitive Effect of 6-Deoxy-D-Glucose (0.06M) on Rate of Oxidation of Glucose-U-C^{14} to $C^{14}O_2$ by Mouse Adipose Tissue at Different Concentrations of Glucose.

Molarity of glucose	% inhibition*
.020	13
.010	43
.005	62
.0025	72

* Inhibition refers to the ratio of rate of glucose oxidation in presence of 6-DOG to rate in its absence, concentration of glucose being the same in both cases. Results are avg values of 2 experiments.

‡ Analysis performed by Elek Micro Analytical Laboratories, Los Angeles.

§ The data presented here are average values for a large series of experiments. The data from control experiments using sucrose, sorbitol, and mannitol are omitted to conserve space. Studies were also carried out with yeast and Ehrlich ascites cells. The reader is referred to the original thesis for detailed studies.

TABLE II. Effects of 6-Deoxy-D-Glucose on Rate of Oxidation of Glucose-U-C^{14} to $C^{14}O_2$ and on Amount of Glucose Derived Carbon Accumulated in Rat Diaphragm. Results expressed as percentage of control.*

Compound	Molarity	$C^{14}O_2$	C^{14} in tissues
6-DOG	.03	81	81
"	.06	74	71
"	.12	48	62
2-DOG	.12	30	78

* % of control refers to rate at which rat diaphragm tissue oxidized 0.01M glucose-U-C^{14} to $C^{14}O_2$ and accumulated C^{14} compounds in the tissue during incubation in presence of the indicated concentration of test compounds relative to rate in absence of the test compound.

inhibits oxidation of glucose and that the inhibition is reversible. The fact that 6-DOG inhibits glucose utilization in mammalian tissue, provides a second 6-position substituted glucose analog for study of glucose metabolism, particularly those steps prior to and including the hexokinase step. This compound offers a means of comparing directly results formerly observed with 6-fluoro-D-glucose.

As far as we are aware, 6-DOG has not been used previously with mammalian tissue as in this study. However, 6-DOG has been used as a tool for studying the mechanism of intestinal absorption of sugars. Crane(4) reported that this sugar is actively absorbed from the intestinal tract, and Wilson *et al.* (5) have reported that glucose inhibits intestinal transport of 6-DOG and the inhibition is reversible.

The evidence presented here provides little information concerning the site or sites of action of 6-DOG in inhibiting glucose oxidation. The observation that 6-DOG is not oxidized to CO_2 in mouse adipose tissue indicates that the observed inhibition is not merely the effect of the tissue metabolizing 6-DOG instead of glucose.

The site (sites) at which 6-DOG blocks glucose metabolism depends largely on whether the analog is altered within the cell. Although 6-DOG cannot be phosphorylated by hexokinase, kinases which will phosphorylate sugars in positions other than the 6-position are known to occur in mammalian tissues(6,7). 2-DOG inhibits glucose metabolism at a multiplicity of sites, due to accumulation of its phosphorylated derivative in the cells. The production of oxidized derivatives of 6-DOG within the cell offers the possibility of these compounds acting as actual blocking agents. However, if 6-DOG is not acted upon by the tissues in which it inhibits glucose oxidation, the most probable points at which metabolism will be blocked are those of cell entry and/or the hexokinase step. 6-DOG has been shown to be an inhibitor of glucose phosphorylation by brain hexokinase(8). At an inhibitor to substrate molar ratio of 100:1, we have found that 6-DOG also competitively inhibits yeast hexokinase by 59%. However, at a ratio consistent with the tissue results, *i.e.*, 12:1, 6-DOG has no effect upon rate of phosphorylation of glucose by yeast hexokinase.

Summary. 6-Deoxy-D-glucose has been shown to be an inhibitor of glucose oxidation in rat kidney slices, mouse adipose tissue, and rat diaphragm. The ratio of 6-DOG to glucose which gave a 50% inhibition of glucose oxidation corresponded to 12:1 in rat kidney slices and rat diaphragm and 4:1 in mouse adipose tissue. The inhibition of glucose oxidation in mouse adipose tissue was shown to be reversible. Experiments using rat diaphragm tissue showed that 6-DOG inhibited uptake of glucose in proportion to decrease in glucose oxidation. Incubation of mouse adipose tissue with 6-DOG uniformly labeled with C^{14} did not result in formation of measurable amounts of $C^{14}O_2$. It is concluded that this compound is incapable of complete oxidation in this tissue. It is believed that the site of competitive inhibition is either at the cell entry mechanism or at the hexokinase reaction. Thus, the metabolic block produced by 6-DOG should be less complex than that produced by the 2-position modified sugars. The use of 6-DOG may be very useful for studying cell permeation mechanisms.

1. Hardegger, E., Montavon, R. M., *Helv. Chim. Acta,* 1946, v29, 1199.

2. Fischer, E., Zach, K., *Chem. Ber.,* 1912, v45, 3761.

3. Woodward, G. E., Cramer, F. B., Hudson,

M. T., *J. Franklin Inst.*, 1953, v256, 577.
4. Crane, R. K., Wilson, T. H., *J. Appl. Physiol.*, 1958, v12, 145.
5. Wilson, T. H., Lin, E. C. C., Landau, B. R., Jorgensen, C. R., *Fed. Proc.*, 1960, v19, 870.
6. Staub, A., Vestling, C. S., *J. Biol. Chem.*, 1951, v191, 395.
7. Hosterlitz, H. W., *Biochem. J.*, 1937, v31, 2217.
8. Crane, R. K., Sols, A., *J. Biol. Chem.*, 1954, v210, 597.

Received April 7, 1961. P.S.E.B.M., 1961, v107.

Isolation and Identification of 17α-Hydroxyprogesterone in Adrenal Venous Plasma of Normal Dogs.* (26637)

BALWANT DAJI AGASHE,† ROBERT G. WEAVER AND KRISTEN B. EIK-NES
(Introduced by Leo T. Samuels)
Departments of Biological Chemistry and Anatomy, College of Medicine, University of Utah, Salt Lake City

Adrenal venous blood of dogs was analyzed for steroids by Farrell and Lamus(1) and 14 different fractions were isolated of which 10 appeared to be steroids. Three of these fractions were characterized as cortisol, corticosterone, and Reichstein's Substance S. Carstensen *et al.*(2) while reporting the isolation of 5-pregnene-3β,17α-diol-20-one (17α-hydroxypregnenolone) in the canine adrenal vein plasma indicated the presence of 4-pregnene-17α-ol-3,20-dione (17α-hydroxyprogesterone) in this fluid but did not characterize the material. The studies referred to were made on dogs under the 'stress' of an acute surgical operation. The present communication deals with the isolation and identification of 17α-hydroxyprogesterone from adrenal venous blood of 'unstressed' normal dogs.

Experimental. a. *Materials.* The animal preparation used for the experiment was that described by Weaver and Eik-Nes(3). An intravenous injection of 25 I.U. of adrenocorticotropin (ACTH) (Upjohn Co.) was given ½ hour before blood was collected from the adrenal pouch into a heparinized tube. No more than 70 to 80 ml of blood were withdrawn per tapping. The plasma was separated from the red cells by centrifugation for 1 hour at 1800 rpm and stored at −15°C in a deep freeze until processed.

Chemicals used were reagent grade. Solvents were purified according to standard procedures(4) and redistilled before use. All evaporations of organic solvents were done under nitrogen at 40°C.

b. *Extraction and purification of the steroids.* The plasma was extracted 4 times using an equal volume of a mixture of ethyl acetate-ethyl ether (1:1 v/v) each time. The extract was washed once with 0.1 volume of 1 N NaOH and 3 times with 0.1 volume of water, and evaporated to dryness. To the residue 0.01 μg of 17α-hydroxyprogesterone-4-C^{14},‡ equivalent to 800 cpm was added to serve as reference standard for detection on paper chromatograms and for calculation of recoveries.

The dry residue was suspended in 20 ml of 70% aqueous methanol and left overnight (15 hours) at −15°C. The suspension was then centrifuged for ½ hour at 1800 rpm in a refrigerated centrifuge and the supernatant decanted and collected(5). The residue was washed with 5 ml of ice-cold 70% methanol, and the wash after centrifugation was combined with the original supernatant. After evaporating to dryness the dilute methanolic extract, the residue was dissolved in 15 ml of n-hexane and washed thrice with equal volumes of 70% methanol (6). The hexane fraction was discarded and the methanolic solution dried down.

* This work was in part supported by Research Grants from U. S. Public Health Service, Bethesda, Md.

† Present address: Dept. of Chemistry, Osmania University, Hyderabad-7, (A.P.) India.

‡ New England Nuclear Corp., Boston, Mass.

For separation of 17α-hydroxyprogesterone from other corticosteroids, the solvent system propylene glycol-methyl cyclohexane was used(7). Whatman No. 1 paper was impregnated with propylene glycol-methanol (2:3 v/v), and excess solution removed by blotting between pads of filter paper. The sample was applied using a mixture of chloroform-methanol (1:1 v/v). The chromatogram was developed for 65-70 hours by the descending method with methyl cyclohexane saturated with propylene glycol, then thoroughly dried for 15 hours in a current of dry air.

The paper chromatogram was scanned for radioactivity in a strip counter,§ and the area of the paper strip showing a peak in radioactivity was cut out and eluted with 10 ml of vacuum distilled methanol. After the solvent was evaporated the residue was purified of paper impurities by dissolving in 15 ml ethyl acetate, washing once with 10 ml 0.5% sulfuric acid (by volume) and twice with 10 ml of water. The ethyl acetate was evaporated and the residue analyzed spectrophotometrically for absorption at 240 mμ using a Beckman Spectrophotometer Model DU with micro cells of 0.3 ml capacity.

c. *Identification of 17α-hydroxyprogesterone.* Material obtained from plasma of several animals showing a running rate in the above mentioned system of chromatography identical with that of authentic 17α-hydroxyprogesterone and exhibiting a distinct absorption at 240 mμ was pooled and further purified for infrared analysis. With corticosterone added as reference compound, the material was chromatographed on paper in a formamide-hexane system for 2 hr over-running, dried for 3 min in air and run in a formamide-chloroform system. As soon as the mobile phase reached almost the bottom of the strip, the chromatogram was removed, solvent front marked and the chromatogram thoroughly dried for 24 hr in a current of air(8). The material behaving chromatographically like 17α-hydroxyprogesterone (R_f 0.88 and R_s 1.33) was eluted, the solvent evaporated and the residue purified as described in step b. The purified extract was rechromatographed on paper in a propylene glycol-toluene system for 4½ hr with testosterone as reference compound. Material exhibiting the running rate of authentic 17α-hydroxyprogesterone (R_s 1.31) was eluted with methanol, solvent evaporated and the product purified by partitioning the material between ethyl acetate and 0.5% sulfuric acid as described earlier.

The material so obtained was further purified by column chromatography on 5 g of Woelm neutral aluminum oxide of activity grade 1. The column was prepared in benzene and developed with benzene and benzene-ether mixtures. Twenty ml eluates were collected as separate fractions. The steroid was eluted with benzene-ether (3:1) mixture. Fifty μg of synthetic 17α-hydroxyprogesterone were also chromatographed on a similar aluminum oxide column. The unknown material and the authentic steroid were subjected to infrared analysis. The spectra were recorded with a Perkin-Elmer Infrared Spectrophotometer, Model 221, employing the potassium bromide technic and a Perkin-Elmer Ultra-micro sampling system(9). The infrared spectrum of the unknown material was identical with that of synthetic 17α-hydroxyprogesterone carried through the last stage of purification (Fig. 1). The peaks in the spectra of the authentic material and the sample, appearing between 4 and 5 μ wave length could be due to some impurity from the aluminum oxide column because this did not appear in the spectrum of the pure, untreated steroid taken in a potassium bromide pellet (Fig. 1).

In these unstressed, ACTH-stimulated dogs an average concentration of 2.2 μg 17α-hydroxyprogesterone/100 ml adrenal venous plasma was found by the measurement of U.V. absorption at 240 mμ and correction for losses on the basis of the ratio of the final radioactivity to that added to the crude extract. Material behaving chromatographically like 17α-hydroxyprogesterone could not be detected in extracts of large pools of systemic blood plasma from the animal preparation used.

§ Nuclear-Chicago, Chicago.

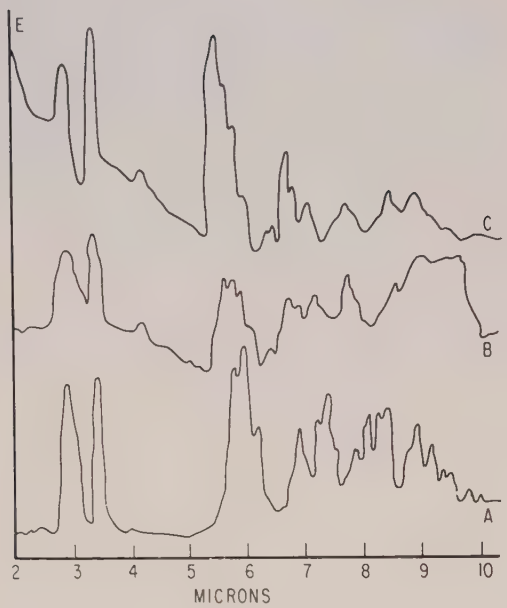


FIG. 1. Infrared spectra of: A, authentic 17α-hydroxyprogesterone; B, authentic 17α-hydroxyprogesterone after chromatography on aluminia oxide; C, compound isolated from adrenal vein plasma.

Discussion. 17α-hydroxyprogesterone was first isolated from adrenal cortical extracts as an inactive corticoid(10,11). Hechter *et al.*(12) and Kushinsky(13) isolated this steroid after perfusion of the bovine adrenal gland with non-labelled and with radioactive progesterone. Plager and Samuels(14) and Levy *et al.*(15) have established the conversion of progesterone-4-C^{14} to 17α-hydroxyprogesterone-4-C^{14} by incubation of adrenal tissue homogenates. In the many studies of steroids in the adrenal vein blood of the dog (1,16,17,18,19) this steroid was neither detected nor characterized. Carstensen *et al.* (2) indicated the presence of this steroid in the adrenal venous effluent of dogs from its partition coefficients in 2 systems of counter-current distribution and its maximal ultraviolet absorption at 240 mμ but did not confirm the indication by more definitive methods.

The fact that this steroid was found in the adrenal venous blood of the dog but not in the circulating blood under similar conditions supports the assumption that it represents a secretory product of the adrenal gland(17, 20). Apparently this steroid undergoes rapid metabolism.

17α-hydroxyprogesterone is an important intermediate in biosynthesis of corticoids. Generally, this steroid has been thought to be metabolized so rapidly in the adrenal gland that it does not leave this organ and appear in significant amounts in the venous blood. By experiment reported here, however, 17α-hydroxyprogesterone has been conclusively proved to be a secretory product of the normal canine adrenal cortex.

Summary. By criteria of paper chromatography in several systems, ultraviolet and infrared spectrophotometry, 4 pregnene-17α-ol-3,20,dione has been isolated and identified in adrenal vein blood of normal dogs given adrenocorticotropin intravenously. This steroid could not be detected in blood from the systemic circulation of dogs given adrenocorticotropin.

The authors wish to express gratitude to Dr. Leo T. Samuels for helpful criticism and to Dr. Gunter Zweig, University of California, Pesticide Residue Research, Davis, Cal., for infrared analysis.

1. Farrell, G. L., Lamus, B., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 89.

2. Carstensen, H., Oertel, G. W., Eik-Nes, K. B., *J. Biol. Chem.*, 1959, v234, 2570.

3. Weaver, R. G., Eik-Nes, K. B., *J. Applied Physiol.*, 1959, v14, 665.

4. Fieser, L. F., *Experiments in Organic Chemistry,* Heath & Co., Boston, Mass., Edition 3, 1959.

5. Zander, J., Simmer, H., *Klin. Wochschr.*, 1954, v32, 529.

6. Reich, H., Nelson, D. H., Zaffaroni, A., *J. Biol. Chem.*, 1950, v187, 411.

7. Savard, K., *ibid.*, 1953, v202, 457.

8. Zaffaroni, A., *Rec. Prog. Horm. Res.*, 1953, v8, 51.

9. Mason, W. B., The Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, March 1958 I Cf. *The Perkin Elmer Instrument News,* 1958, v10, 1.

10. Pfiffner, J. J., North, H. B., *J. Biol. Chem.*, 1940, v132, 459.

11. Euw, von J., Reichstein, T., *Helv. chim. Acta.*, 1941, v24, 879.

12. Hechter, O., Zaffaroni, A., Jacobson, R. P., Levy, H., Jeanloz, R. W., Schenker, V., Pincus, G., *Rec. Prog. Horm. Res.*, 1951, v6, 215.

13. Kushinsky, S., The Isolation of Products from

Bovine Adrenal Perfusion with Progesterone and Progesterone-4-C^{14}, Doctoral Thesis, Boston Univ., Boston, Mass., 1955.

14. Plager, J. E., Samuels, L. T., *J. Biol. Chem.*, 1954, v211, 21.

15. Levy, H., Jeanloz, R. W., Jacobson, R. P., Hechter, O., Schenker, V., Pincus, G., *ibid.*, 1954, v211, 867.

16. Zaffaroni, A., Burton, R. B., *Arch. Biochem. & Biophys.*, 1954, v42, 1.

17. Bush, I. E., *Ciba Found. Coll. Endocrinol.*, 1953, v7, 210.

18. Farrell, G. L., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 338.

19. Hechter, O., Macchi, I. A., Korman, H., Frank, E. D., Frank, H. A., *Am. J. Physiol.*, 1955, v182, 29.

20. Hechter, O., Pincus, G., *Physiol. Rev.*, 1954, v34, 459.

Received April 10, 1961. P.S.E.B.M., 1961, v107.

Triiodothyronine Uptake by Erythrocytes in Mongolism. (26638)

EUGENE JACKIM, JOSEPH WORTIS, AND JEROME ADESMAN

Division of Pediatric Psychiatry and Radioisotope Laboratory, Jewish Hospital of Brooklyn, N. Y.

In view of the recent discovery that mongolism is associated with a chromosomal anomaly, there is good reason to believe(1) that certain enzyme anomalies also exist. During a series of experiments on enzyme systems in mongolism we have observed that red blood cells of mongoloid subjects tend to have a higher oxygen uptake and lower rate of phosphorylation than control subjects(2). In an attempt to explain this phenomenon, we decided to follow up the suggestion of Kurland *et al.*(3) who noted in a series of 9 cases that mongoloid rbc had a "tendency" to a higher triiodothyronine uptake *in vitro*. Our present studies show that this high triiodothyronine uptake is highly characteristic of mongolism.

The method used was the standard radioiodinated triiodothyronine *in vitro* rbc uptake test of Hamolsky, Golodetz and Freedberg(4). We used 23 euthyroid individuals, both male and female, as controls. Their normality was established by one or more of the following criteria: 1) lack of thyroid disorder symptoms, 2) normal protein-bound iodine, and 3) normal neck iodine^{131} uptake. Since some of these controls were individuals being tested for thyroid malfunction, the number of borderline cases is probably somewhat higher than might be found in a random normal population. The mongoloid group consisted of 22 individuals, 12 of which were out-patient mongoloid children ranging in age from 5 to 11 years, 5 mongoloid infants under one year of age, and 5 institutionalized mongoloid adults.* A small group of non-mongoloid retarded children and another group of institutionalized adult psychiatric cases, mostly schizophrenics, were studied for comparative purposes. Abnormal thyroid activity due to medication was ruled out in all cases.

It was found that the triiodothyronine uptake increase was almost invariably present in the rbc of the mongoloid subjects. The mean for the control group, consisting of 23 samples, was 15.4, with a range between 11.8 and 19.3. The mongoloid group, consisting of 22 samples, had a mean value of 22.9, with a range between 13.9 and 29.8. Only 2 mongoloid values fell into the normal range. One of these was a borderline value, while the other was that of an obese borderline hypothyroid mongoloid with a PBI of 3.2. Values in female subjects tended to be slightly lower. When the 2 sexes were considered separately, the borderline hypothyroid mongoloid mentioned above was the only mongoloid subject falling into the normal range (Fig. 1). When the adult psychiatric cases were used as controls a slight

* This work was supported by the M. J. Solomon Fellowship Fund, and by an Anonymous Donor. Thanks are due to Letchworth Village, N. Y., and to Brooklyn State Hospital for cooperation in supplying specimens.

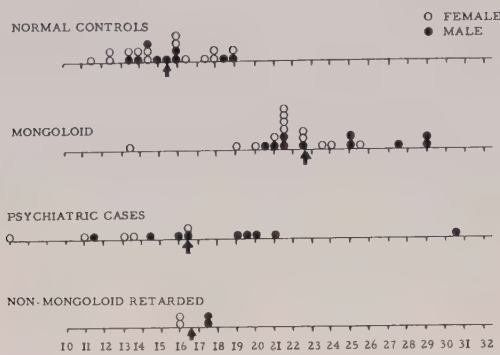


FIG. 1. Triiodothyronine uptake values of erythrocytes, expressed in percentages.

overlapping of values was encountered. Though no correlation with age has been noted by other workers or by us, we found a single remarkably high value in a senile psychotic patient 90 years old.

Summary. The chromosomal anomaly of mongolism suggests the likelihood of enzymatic anomalies. In a series of enzymatic studies in mongolism a tendency to high oxygen uptake and low phosphorylation rate in the red blood cells was encountered. In the present study a high triiodothyronine uptake of rbc was found, which throws light on both the high respiratory rate and the uncoupling of phosphorylation. The range of high thyroid hormone uptake values was almost completely outside the range of normal controls, though a few adult psychiatric cases were found with slightly elevated values.

1. Jacobs, P., Baikie, A. G., Court Brown, W. M., Strong, J. A., *Lancet,* 1959, v1, 710.

2. Wortis, J., Jackim, E., *Recent Advances Biol. Psychiat.,* 1961, v3, 185.

3. Kurland, G. S., Fishman, J., Hamolsky, M. W., Freedberg, A. S., *J. Clin. Endocrin.,* 1957, v17, 552.

4. Hamolsky, M. W., Golodetz, A., Freedberg, A. S., *ibid.,* 1959, v19, 103.

Received April 10, 1961. P.S.E.B.M., 1961, v107.

Tolerance to Bacterial Endotoxin Produced by Proliferation of Gram Negative Bacteria in the Kidney. (26639)

WILLIAM R. MCCABE* (Introduced by Paul Heller)

Veterans Administration West Side Hospital and University of Illinois College of Medicine, Chicago

Tolerance to the toxic effects of endotoxins of Gram negative bacteria has been demonstrated only after repeated injections of dead Gram negative bacteria or their products, but not during the course of clinical or experimental infections(1,2,3). Previous clinical observations by the author suggested that tolerance to bacterial endotoxin did occur during the course of human pyelonephritis and prompted investigation of the effect of experimental pyelonephritis on the pyrogenic response to endotoxins. This preliminary report is based upon the initial observations made during this study.

Material and methods. Unilateral pyelonephritis was produced in six 2.0-3.0 kg male New Zealand rabbits by intravenous injection of 1×10^9 *E. coli* (0-111:B-4), followed immediately by occlusion of the right ureter for 36 hours. A loose ligature had been placed about the ureter previously through a right flank surgical approach and fixed externally for subsequent tightening. Six weight matched control rabbits underwent a similar operative procedure without ureteral occlusion and received an equivalent endotoxin challenge of 1×10^9 formalin killed *E. coli* (0-111:B-4) on the same day. The pyrogenic response of the control and pyelonephritic rabbits to 0.5 μg of purified *S. enteriditis* lipopolysaccharide (Difco) was determined 10 to 21 days later.

The rabbits were maintained in wire cages in an air conditioned room without restraint during the experimental period. Two hours were allowed for acclimatization and determination of base line temperatures. *S. enteriditis* endotoxin was injected in the marginal ear vein and temperatures were deter-

* Veterans Administration Clinical Investigator.

TABLE I. Fever Indices in Rabbits Given 0.5 γ *S. enteriditis* Endotoxin.

	Rabbits with pyelonephritis	Control rabbits
	7	46
	16	34
	19	52
	4	42
	17	51
	15	46
Mean	13 ± 5.5	45 ± 6.01
$T_{10} = 8.78, P < .001$		

mined every 30 minutes for an additional 6½ hours. Temperatures were recorded on a Yellow Springs Telethermometer using thermocouples implanted deeply in the paraspinous muscles. Temperature curves were plotted on 1.0 cm graph paper with each centimeter representing 30 minutes and 0.5° (F.) temperature. The area above the base line was measured by an Ott Universal Planimeter and this reading was recorded as the fever index.

All glassware was heated at 180°C for 3 hours prior to use. The endotoxin was suspended in pyrogen free saline (Abbott) for injection.

Results. The rabbits in whom pyelonephritis was produced were lethargic and febrile for 3 to 4 days after injection of viable *E. coli.* The control rabbits exhibited similar symptoms for 18 to 24 hours after injection of dead bacteria. Both groups of animals had normal temperatures (<103°F.), were active, and appeared healthy at time of challenge with purified endotoxin.

The challenge dose of 0.5 μg of *S. enteriditis* lipopolysaccharide routinely produced a striking temperature elevation with a maximum rise of 3°F or more and the fever persisted for 6 hours or longer in all control animals. Fever indices of 34 units or more were observed in control animals. The mean fever index for this group was 45 ± 6.0 units.

In contrast (Table I), rabbits with pyelonephritis had maximum temperature elevations of less than 3°F and the temperature always returned to the preinjection level within 4½ hours. The mean fever index (13 ± 5.5 units) in the 6 rabbits with pyelonephritis was significantly lower than that observed in the controls ($T_{10} = 8.78$, $P < .001$).

In addition to the significant difference in magnitude and duration of fever of the 2 groups the pattern of the fever curves was strikingly different. Fig. 1 illustrates the fever curves of a control rabbit and a rabbit with pyelonephritis. The control animals had an initial febrile peak followed by another temperature rise exceeding the initial fever peak 2½ to 3½ hours after administration of endotoxin. This is typical of the pyrogenic response to endotoxin in rabbits. The fever curve of the pyelonephritic rabbit exemplifies the response in endotoxin tolerant rabbits. The initial febrile reaction is essentially unaltered but is followed by a prompt return to base line levels. In all rabbits with pyelonephritis the maximum temperature elevation was observed within 1½ hours after endotoxin administration while maximum temperature always occurred more than 2½ hours after endotoxin challenge in the controls.

Catheterized or post mortem bladder urine from the pyelonephritic rabbits contained *E. coli.* Necropsy revealed moderate unilateral hydronephrosis and histologic pyelonephritis in 2 rabbits and unilateral pyonephrosis in 2 others. *E. coli* was cultured from the right kidney of these 4 animals. Two

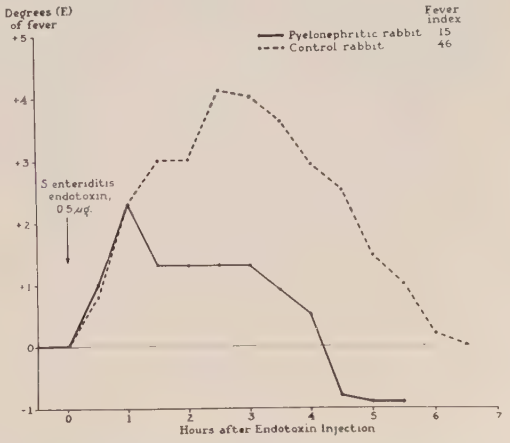


FIG. 1. Fever curves observed in a control rabbit and a rabbit with pyelonephritis after administration of 0.5 μg of *S. enteriditis* endotoxin. Similar curves were observed in the other control and pyelonephritic rabbits.

pyelonephritic rabbits were saved for further investigation. Cultures of the urine and kidneys were sterile and no gross or microscopic renal abnormalities were observed in the control rabbits.

Discussion. Although it has been well recognized that tolerance to endotoxin may be readily induced it had not previously been observed to result from actual bacterial infection(2,3). The present study indicates that infection of the kidney by Gram negative bacteria is capable of inducing tolerance to heterologous endotoxin. A previous study failed to demonstrate pyrogen tolerance following experimental *E. coli* peritonitis in rabbits(2), but these apparently conflicting observations may reflect only a difference in the 2 types of infection. The experimental peritoneal infection was acute and the animals either succumbed rapidly or recovered without evidence of persistent chronic infection. Pyelonephritis, on the other hand, was associated with bacterial proliferation which continued to the time of endotoxin challenge. The latter infection allowed more prolonged exposure of the reticuloendothelial system to endotoxin. This agrees with previous observations that repeated prolonged endotoxin challenge produces tolerance more effectively than a brief exposure.

1. Beeson, P. B., *J. Exp. Med.*, 1947, v86, 29.
2. Bennett, I. L., *ibid.*, 1948, v88, 267.
3. Heyman, A., Beeson, P. B., *J. Lab. & Clin. Med.*, 1949, v34, 1400.

Received April 17, 1961. P.S.E.B.M., 1961, v107.

Abnormal Palate Morphogenesis in Mouse Embryos Induced by Riboflavin Deficiency.* (26640)

BRUCE E. WALKER AND BURTON CRAIN, JR.
Department of Anatomy, Univ. of Texas Medical Branch, Galvestion

Kalter and Warkany(1) produced a variety of anomalies in the offspring of pregnant mice by feeding a riboflavin-deficient diet containing galactoflavin (a riboflavin antagonist). The frequency of the various anomalies differed among the several inbred strains used. Cleft palates occurred in 76% of the offspring from treated mice of the DBA strain. Embryonic development was not investigated by these authors, so the following study was undertaken to search for a critical deviation in palate morphogenesis during induction of riboflavin deficiency.

Materials and methods. Pregnant mice of the DBA and C57BL strains were fed a riboflavin-deficient diet containing 12 mg galactoflavin† per g of diet, starting at 10⅓ days postconception and transferring to regular diet at 14⅓ days. Uteri were collected at 14 to 16 days postconception or at 18⅓ days, fixed in Bouin's fluid, and the embryos dissected out for palate examination and for estimates of "developmental age" according to their stage of morphological development (2).

Results. Treated litters of the DBA strain at 14 to 16 days postconception differed grossly from untreated litters in 2 respects. First, the treated embryos were quite edematous; second, the long axis of the snout was usually at right angles to the body axis instead of being pressed down on the chest as in the control embryos.

Table I shows the condition of palate morphology(3) relative to the embryo's morphological rating, the latter calculation being based on the developmental state of several external features(2). The palates of all control embryos had moved to the horizontal plane and started to fuse by morphological rating 11 in this experiment and by 12 in a previous experiment(3). Some of the

* Supported by a research grant from Nat. Inst. of Dental Research and a Traineeship from USPHS.

† Supplied by Merck and Co., Inc., Rahway, N. J. through the courtesy of Dr. N. S. Ritter.

TABLE I. Relation of Palate Stage to Morphological Rating in Embryos from Riboflavin-Deficient (R) and Control (C) Pregnant Mice of the DBA Strain.

Morphological rating	Palate stage 1		2		3		4		5		6		7	
	R	C	R	C	R	C	R	C	R	C	R	C	R	C
−2	3													
−1	2	1												
0	5	1												
1	2	1												
2	1													
3	4													
4	5	4												
5	6	3												
6	3	1												
7	12	3												
8	3						3	2	2	2				
9	5					1			1	2	1			
10	3					1	1		1	1	3	3	1	1
11			1				1				6	3	1	1
12	7						2		1		2	2	2	2
13					2		1				3		5	5
14	2		1				1						2	1
15	2				2								3	1
16	2						1						4	2
17							2						3	3
18	2				2		1						4	4
19							1				1		3	3
20													1	
21											1			
22					1									
23	1				2									
24					1								1	
25														
26														
27														
28													1	
29														

treated embryos had also commenced palate fusion by morphological rating 11, but most of them showed some degree of retardation in movement of the palatine shelves from a vertical to a horizontal position (Table I). However, aside from this alteration in shelf position, no pronounced morphological changes of the palatine shelves were seen grossly or in sections.

At 18 days postconception, the morphology of the fetal palates was as follows: both shelves vertical in 6 fetuses; one shelf vertical and the other horizontal in 7 fetuses; both shelves horizontal in 10 shelves horizontal and fused (normal) in 31 fetuses. This constitutes an average palate stage(2) of 0.80 and a cleft palate frequency of 42%.

There were no cleft palates among 67 fetuses collected at day 18 from 11 treated C57BL female mice, so no litters were collected at the time of palate closure in this strain.

Discussion. The observations on effects of riboflavin deficiency in this experiment support the conclusions of Kalter and Warkany(1) that there are strain differences in the effects of this treatment and that a high frequency of cleft palate can be induced in offspring of the DBA strain. In the embryos from treated DBA mice, the only observed deviation in palate morphogenesis of sufficient magnitude to cause cleft palate was retardation of palatine shelf movement. At the time of normal palate closure, no difference was seen in the morphology of palatine shelves in treated embryos which achieved shelf movement within the normal period of time and those with delayed shelf movement. Subsequent alterations in the morphology of the palate region were apparently secondary

to failure of shelf fusion. In untreated embryos of 3 strains previously investigated(3), palatine shelves had reached the horizontal position and started to fuse before a morphological rating of 13 in all cases. It is probable that embryos in which shelf fusion had not started by morphological rating 15 would develop cleft palate. In Table I, 20 embryos had unfused palates and 22 had fused palates at morphological rating 15 or later. This frequency of delayed shelf movement is similar to the frequency of cleft palate in the late (day 18) fetus. The delay in shelf movement was also reflected in the various shelf positions in the fetus.

Thus, the sensitive phase of palate morphogenesis is again the process of shelf movement, as it was with cortisone-induced cleft palate(4), hypervitaminosis A-induced cleft palate(2), the spontaneous cleft lip-cleft palate of the A/Jax strain(5) and cleft palate produced by amniotic sac puncture(6). The cause of shelf movement retardation was not pressure against the lower jaw and tongue due to the head being forced against the chest as in the case of amniotic sac puncture(6), since the lower jaw was not in contact with the chest. However, the possibility of pressure effects cannot be ruled out since the edematous condition of the embryo may have altered normal pressure relationships in the amniotic cavity and embryo.

Summary. Retardation of palatine shelf movement was found to be the morphogenetic basis for the cleft palates induced in DBA strain mouse embryos by a riboflavin-deficient, galactoflavin-containing diet.

1. Kalter, H., Warkany, J., *J. Exp. Zool.*, 1957, v136, 531.

2. Walker, B. E., Crain, B., Jr., *Am. J. Anat.*, 1960, v107, 49.

3. Walker, B. E., Fraser, F. C., *J. Embryol. Exp. Morph.*, 1956, v4, 176.

4. ———, *ibid.*, 1957, v5, 201.

5. Walker, B. E., Crain, B., Jr., *Tex. Rep. Biol. Med.*, 1959, v17, 637.

6. Walker, B. E., *Science*, 1959, v130, 981.

Received April 17, 1961. P.S.E.B.M., 1961, v107.

Quantitative Changes of a Human Salivary Bactericidin for Lactobacilli Associated with Age.* (26641)

LLOYD B. AUSTIN AND BERNARD J. ZELDOW (Introduced by J. J. Holland)
School of Dentistry, University of Washington, Seattle

It has been established that the oral microbial flora are relatively characteristic for an individual. Antibacterial systems present in the saliva are among several factors postulated to contribute to selection of the resident oral flora. Since selection of the oral microbiota usually commences shortly after birth, the influences exerted by such systems would be expected early. The salivary glands of human infants, however, are regarded as physiologically immature(1) and it is possible, therefore, that the level of any salivary antibacterial system would also reflect this immaturity.

A bactericidin for various species of lactobacilli has been described in mixed, parotid and submaxillary saliva of human adults(2). Since this is not a product of bacteria and is lacking in the serum, it appears to be an *endogenous* product of the salivary glands. The present report gives results of an investigation of the titers of this salivary lactobacillus bactericidin as a function of age.

Methods. The mixed salivas were collected from humans ranging in age from birth to 56 years. Individual salivas were grouped together for study as follows:

A. Premature newborns: delivered prior to expected gestation and weighing 5½ lb or less
B. Full-term newborns: age 1 to 5 days
C. Infants: age 5 days to 1 year
D. Preschool children: age 1 to 6 years

* Supported by grant from N.I.H., U.S.P.H.S.

E. Primary school children: age 6 to 12 years

F. Adults: age 20 to 56 years

All subjects beyond one year of age were placed into their respective group by calculating to the nearest birthday.

Saliva from the adult and primary school age children was collected by paraffin stimulation and expectorating the accumulated saliva into a sterile jar. In the case of preschool age children, infants and the full-term newborns, a sterile ball of cotton firmly attached with a string was inserted into the oral cavity with the aid of a tongue blade, permitting the secretions to collect in the cotton. When saturated, it was removed by the string and the saliva was expressed with a sterile hand press into a sterile receptacle. It was found that this method of collection had no deleterious effect upon the antibacterial system investigated.

The bactericidal activity of the individual salivas was measured using the assay organism, *Lactobacillus acidophilus* ATCC 4357. All specimens with the exception of those collected from the premature newborn group were assayed by the 2-fold dilution method in Lactobacillus Selective broth pH 5.5(3), to which 1/5 volume of a suspension of the assay organism containing approximately 3×10^7 organisms per ml was added. The mixtures were incubated at 37°C for 18 hours and examined for turbidity as evidence of growth. For quantitative comparison, bactericidal activity is expressed in units representing double the reciprocal of the maximum number of times 0.5 ml of saliva could be diluted and still inhibit the growth of the assay organism. Four units of activity per ml is the minimum activity discerned by this method.

Saliva from the premature newborn was collected by inserting a pledget of cotton into the mouth with a hemostat and absorbing the salivary secretions. The antilactobacillus activity of these specimens was immediately tested by placing the saturated cotton pledget on a pour plate seeded with the assay organism. After 18 hours incubation at 37°C, activity by this qualitative agar diffusion method was discerned by a clear zone representing inhibition of the organism.

TABLE I. Number of Subjects in Varying Age Groups Showing Indicated Levels of a Human Salivary Lactobacillus Bactericidin.

Levels of bactericidin activity (units/ml)	Groups					
	A*	B	C	D	E	F
	No. of subjects					
0	4					
<4†		15	0	0	0	0
4		4	11	2	0	0
8		2	10	16	2	1
16		0	5	12	18	11
32		0	0	9	10	7
64		0	0	0	1	2

* Agar diffusion method applicable to Group A only.

† <4 indicates no activity by quantitative bactericidin test.

Results. The data are presented in Table I. One hundred forty-two specimens of mixed saliva were studied. Although limited in number, the results strongly suggest that the lactobacillus bactericidin system is lacking in the saliva of the premature infants. Moreover, during the first 4 days of life the majority of full-term newborns showed no salivary antilactobacillus activity as discerned by the quantitative lactobacillus bactericidin test. However, the appearance of the lactobacillus bactericidin is rapid. After 4 days of life the salivas of all of the full-term infants demonstrated activity. With increasing age, there was an increase in amount of the lactobacillus bactericidin in the saliva. A chi square test of the frequency distributions of subjects according to levels of lactobacillus bactericidin shows significant differences at the .001 level in Groups B, C, D and E. The pattern of distributions reveals systematic shift of increasing activity with increasing age until the adult level is reached. Apparently, it is not until 6 to 12 years of age that the adult level is approached, there being no significant difference in the frequency distribution of subjects in Groups E and F.

The lactobacillus bactericidin of the saliva of infants and children possessed all the known qualities of the adult system(2,4); namely, it required a dialyzable heat stable anionic cofactor, and the non-dialyzable fraction was destroyed at 75°C for 5 minutes at

pH values in the range of 7.2 to 11.0 but was unaffected by such treatment at pH 5.5. Moreover, the bactericidin is not adsorbed by the homologous organisms but is adsorbed on bentonite. Comparison of the various groups indicated that the same system appeared at the various age levels.

Discussion. The correlation between increase of age and increasing levels of salivary lactobacillus bactericidin in infants and children does not differ from that which has been demonstrated with other biological systems. For example, the amylase content of saliva has been shown to increase with age (5,6). Moreover, it has been reported that the elevated sodium(7,8) and chloride ion(9) levels of infants decrease as the individual passes to adulthood. Humans have long been considered to be deficient in immunologic capacity during the neonatal period (10,11,12). The explanation for these findings is open to speculation, but maturation of the tissues involved should be considered.

A survey of the literature indicates that there may be several salivary antilactobacillus systems(2,13). Because of this, it was necessary to characterize the system under investigation. The data presented regarding pH range, heat stability, dialysis and adsorption suggest that this antilactobacillus system is markedly similar to the one previously described by Zeldow(2,4) and Kerr and Wedderburn(14). A comparison between the various groups also indicates that the same system appeared at the various age levels.

Because of the time of first appearance and the quantitative increases in the lactobacillus bactericidin with increasing age, it is evident that the role played by this system is probably initiated at a very early age.

Summary. 1. The results strongly suggest that the lactobacillus bactericidin system is not present in saliva of premature newborns. 2. Within the first 4 days of life the majority of full-term newborns show no salivary antilactobacillus activity. 3. There is a systematic increase of lactobacillus bactericidin titers until the adult group's range of activity is approximated, which occurs at about the first decade of life. 4. With the exception of the premature newborns, all age groups show a range of activity. 5. The lactobacillus bactericidin studied was characterized with respect to pH range, dialysis, heat stability and adsorption.

1. Burgen, A. S.V., *Canad. Dent. A. J.,* 1960, v26, 258.

2. Zeldow, B. J., *J. Dent. Res.,* 1959, v38, 798.

3. Rogosa, M., Mitchell, J. A., Wiseman, R. F., *J. Bact.,* 1951, v62, 132.

4. Zeldow, B. J., *J. Dent. Res.,* 1961, in press.

5. Nicory, C., *Biochem. J.,* 1922, v16, 387.

6. Mayer, W. B., *Bull. Johns Hopkins Hosp.,* 1929, v44, 246.

7. Prader, A., Gauteir, E., Gauteir, R., Naef, D., *Helv. paediat. Acta,* 1955, v10, 29.

8. Hungerland, H., Quenzlern, J., Weber, H., *Z. Kinderheilk,* 1957, v80, 179.

9. Dances, J., Grobow, E., Boyer, A., *J. Pediat.,* 1957, v50, 459.

10. Baumgartner, L., *Yale J. Biol. Med.,* 1934, v6, 403.

11. Osborn, J. J., Dances, J., Julia, J. F., *Pediatrics,* 1952, v9, 736.

12. Bridges, R. A., Condie, R. M., Zak, S. J., Good, R. A., *J. Lab. Clin. Med.,* 1959, v53, 331.

13. Green, G. E., *J. Dent. Res.,* 1959, v38, 262.

14. Kerr, A. C., Wedderburn, D. L., *Brit. Dent. J.,* 1958, v105, 321.

Received April 20, 1961. P.S.E.B.M., 1961, v107.

Particle Counting of Polyoma Virus.* (26642)

KENDALL O. SMITH AND MATILDA BENYESH-MELNICK
Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas

There is a need for combined physical and biological quantitation of polyoma virus, particularly as we wish to determine the variations in the ratio of the number of physical virus particles to the number of infectious units in polyoma-infected cell cultures during various phases of the growth cycle. Polyoma virus is often extensively aggregated when observed in the electron microscope. This feature may frustrate accurate estimation of total virus mass by biological titrations alone. The present study describes a method for counting polyoma virus particles, and shows a correlation between physical and biological measurements on similar preparations.

Materials and methods. A plaque-purified polyoma virus strain (obtained from Dr. R. Dulbecco, California Inst. of Technology) was used. The strain was maintained by serial passages in primary or secondary Swiss mouse embryo cultures, according to the methods described from Dulbecco's laboratory(1,2). With passage, the virus maintained its cytopathogenic activity in mouse embryo cultures, its ability to agglutinate guinea pig red blood cells, and its capacity to be neutralized by specific polyoma antiserum (obtained from Dr. B. Eddy, Nat. Inst. of Health).

The plaque assay described by Dulbecco and Freeman(1) was employed with several modifications. A 2-hour adsorption period at 37°C was used and the virus inoculum was in a volume of 0.5 ml, since using smaller amounts of fluid caused drying of the center portion of the monolayers. The overlay medium contained 5% horse or fetal bovine serum.

Guinea pig red blood cells, collected in Alsever's solution, washed 3-4 times in veronal buffer (pH 7.3) and kept at 4°C up to 4 days, were used for the hemagglutination (HA) test. They were washed once more before use. Serial 2-fold dilutions of virus were made in veronal buffer (pH 7.3) in transparent plastic panels (Linbro); 0.5 ml of 0.5% red cell suspension was added to 0.5 ml of the virus dilutions and the panels kept overnight at 4°C. The HA titers were expressed as the reciprocal of the last dilution of virus eliciting complete agglutination.

The basic technic used for virus particle counting was that described by Sharp(3) and Smith and Sharp(4) for vaccinia virus. Some modifications were made to facilitate the counting of the much smaller polyoma virus (45 mμ). Centrifugation for counting was done in the Spinco SW-25.1 swinging bucket rotor at 25,000 rpm. Maximum centrifugal force achieved with the SW-25.1 rotor is 90,000 × G, which gives rapid sedimentation of polyoma virus from a short column of fluid. Flat bottomed lucite chambers were made for the cups so that flat agar plugs could be inserted and removed easily. Fig. 1 shows the components used in this procedure. The lucite adapter (A) contains a flat bottomed cylindrical chamber 19 mm deep and 19 mm in diameter. Into this chamber slips a snugly fitting lucite insert (B) into which a pre-cut agar disc (C) of approximately 13 mm diameter has been placed. Agar is cut with an ordinary cork hole borer. A small air escape hole is cut on one edge of the agar disc. The parts are assembled wet so that all spaces between components are filled with distilled water. One ml of the appropriately diluted virus suspension is placed in the chamber, centrifuged for a sufficient time (approximately 20 minutes), the supernatant fluid carefully removed by suction, the lucite insert removed with a tapered lucite rod (D), the agar block lifted out with a curved spatula (E) and placed on a slide to dry. The remaining steps of preparing the pseudoreplica for examination in the electron microscope are identical to those previously described(3).

* Aided by grants from Nat. Cancer Inst., and National Inst. of Allergy and Infect. Dis., Nat. Inst. Health, U.S.P.H.S.

FIG. 1. Apparatus used in polyoma virus counting.

Calculation of number of particles of virus per ml of original fluid depends upon the following considerations. 1. Approximately 93% of the virus is recovered on the first pseudoreplica. A correction factor of 1.07 is used. 2. The chambers used in this work were cylindrical rather than sectoral in shape. At the zone of virus sedimentation, therefore, there is a slight radial spread of virus particles moving to the flat agar receiving surface. This effect produces a dilution of approximately 7%. A correction factor of 1.07 is used. (The short column of virus suspension minimizes this effect. However, sectoral chambers are being designed to eliminate this consideration.) 3. At intervals a calibrated carbon replica grating (obtained from Ernest F. Fullam, Inc.) is photographed at the magnification routinely employed for particle counting (4,600) and projected on the gridded screen used for counting. Projection magnification is approximately 20 $\times$. The area examined on each preparation is thus calculated directly. The area usually examined per micrograph is 50 to 70 μ^2. A total of 5 micrographs are examined for a single calculation.

Purified crystalline trypsin (Carworth) and chymotrypsin, delta (Nutritional Biochemicals Corp.) were used. Virus-enzyme mixtures were incubated at 37°C for 20 minutes. After enzyme treatment specimens were treated with sonic vibration for 30 seconds in a manner described previously(4). Filtered saline was used as a diluent.

Electron microscopy was done with an RCA Model EMU-3-C. Low speed centrifugation was done in an International horizontal centrifuge at 165 $\times$ G for 10 minutes in conical centrifuge tubes.

Results. Various concentrations of enzyme mixture were used to clarify crude virus suspensions for counting. Some reciprocity between the effects of time and enzyme concentration was noted. Treatment for prolonged periods of time resulted in a loss of countable virus particles. Final enzyme concentrations of 0.01-0.033% were found satisfactory for treatment at 37°C for 20 minutes.

Fig. 2 shows fields of polyoma virus typical of those counted. The particles were quite uniform in size and morphology. Aggregation was one of the notable features in these preparations, however. Some preparations showed numerous doughnut shaped forms, apparently empty membranes (see arrow, Fig. 2). In many preparations filamentous structures were a prominent feature (Fig. 3). These forms were not counted in this study.

Experiments were done to determine whether the particle counting methods as described above are valid for quantitating polyoma virus. Fig. 4 shows the results of one of these experiments. Serial dilutions of treated virus were made and each dilution counted. The average number of particles per field is plotted against relative concentration. The straight line drawn through these points goes through the origin.

The precision of the method was studied by making repeated counts on the same specimens. Many of these determinations were made on different days. The results are shown in Tables I and II. Average deviation

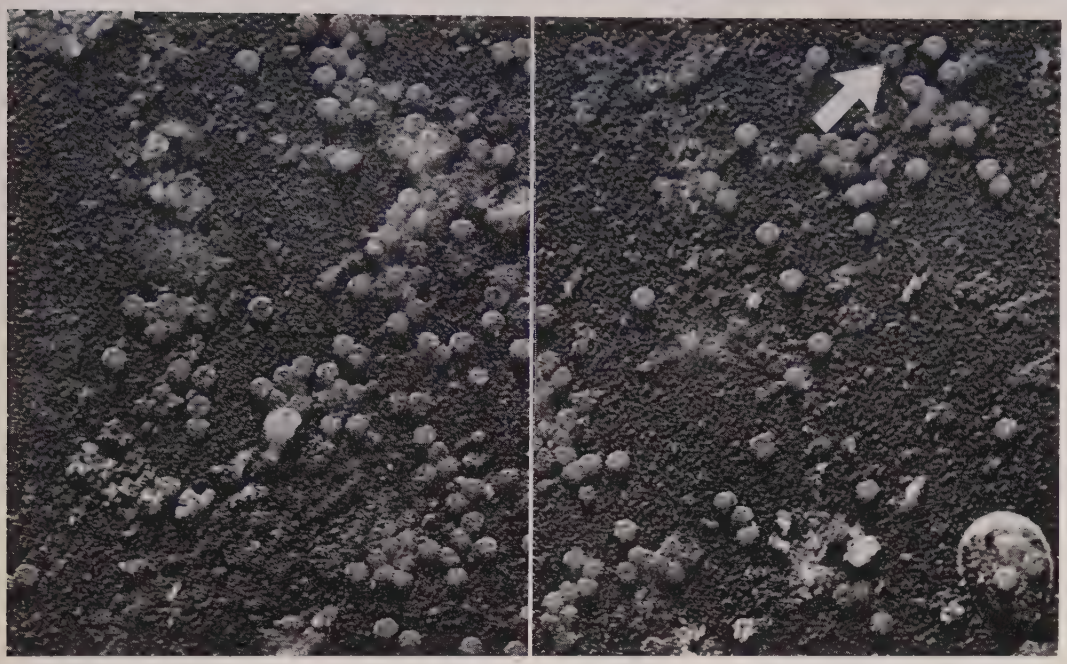


FIG. 2. Typical fields of polyoma virus examined for particle counting (66,000 ×). Arrow indicates doughnut form.

FIG. 3. Field showing filamentous structure found in many polyoma preparations (44,000 ×).

was 16 and 17% for 2 different specimens.

We are particularly interested in combining biological and physical methods in the analysis of virus-cell interaction phenomena, considering especially the cytocidal and integrated characteristics of this virus. One of the first efforts in this direction was to correlate virus characteristics using virus suspensions harvested at different intervals during a growth curve experiment with secondary mouse embryo cells infected with a high virus multiplicity.

The samples described in Table III were taken 4-8 days after infection. They contained both intra- and extracellular virus obtained by 4 cycles of freezing and thawing of the infected cultures without centrifuga-

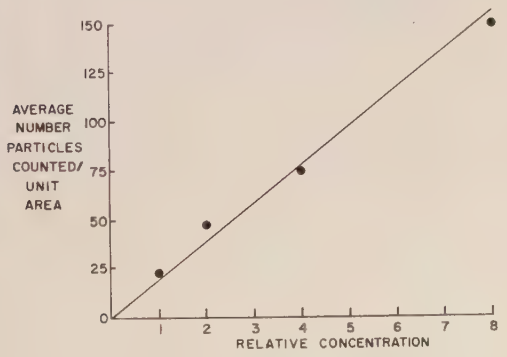


FIG. 4. Relationship between serial dilution and particle counts of polyoma virus.

TABLE I. Precision of Polyoma Particle Counting; Virus Not Pre-centrifuged at Low Speed.

No.	Avg No. particles/unit area	Calculated No. particles/ml ($\times 10^9$)	% deviation from avg
1	211	5.0	21
2	304	7.2	14
3	318	7.5	19
4	261	6.2	2
5	259	6.1	3
6	379	8.9	41
7	204	4.8	24
8	236	5.6	11
9	266	6.3	0
10	213	5.0	21
Avg	265	6.3	16

tion of the cell debris prior to examination. The average particle:plaque forming unit (PFU) ratio was 200. An average of 7×10^7 particles and 4×10^5 PFU was equivalent to one hemagglutination unit. Table IV

TABLE II. Precision of Polyoma Particle Counting; Virus Pre-centrifuged at Low Speed.

No.	Avg No. particles/unit area	Calculated No. particles/ml ($\times 10^9$)	% deviation from avg
1	160	3.8	19
2	174	4.1	12
3	179	4.2	10
4	246	5.8	24
5	230	5.4	16
6	228	5.4	21
7	154	3.7	17
Avg	196	4.6	17

describes the same characteristics of samples containing extracellular virus alone, obtained by low speed centrifugation of supernatant fluids to remove cell-associated virus and large virus aggregates. The particle:PFU ratio was much lower, averaging 38. An average of 5×10^6 particles and 1.4×10^5 PFU was equivalent to one hemagglutination unit. These values are 5-10 fold less than those found for whole, uncentrifuged virus suspensions.

Similar experiments were done also with other supernatant fluids obtained from infected cultures when CPE was extensive and a large number of cells had come free from the glass. Counts and titrations were made on this and on supernatant fluids after low speed centrifugation. Table V shows the results of 2 experiments. Low speed centrifugation removed 83-92% of the total number of virus particles, the majority of which were obviously in large aggregates. Fortunately the enzyme and sonic treatment of these uncentrifuged suspensions reduces these aggregates to sizes sufficiently small for counting. It was not uncommon to see aggregates of 50-100 particles on microscopic examination of these preparations, however. It is apparent, then, that the infectivity and hemagglutination titers of these preparations were not significantly reduced by low speed centrifugation, although the particle concentration was greatly reduced.

Discussion. A relatively simple, precise technic for counting polyoma virus particles in tissue culture fluids heavily contaminated with cell debris is described. At least 3 morphologic types of structures were observed: (a) spherical, electron dense particles, (b) flat, electron transparent "doughnut" forms, and (c) filaments. We have consistently observed filamentous structures in high titer material. The diameter of the filaments is very nearly that of the spherical particles. Occasionally a filament was seen with internal structure, apparently segmenting into spherical particles. We agree with Howatson and Almeida(5) that these are

TABLE III. Particle Count—Infectivity—Hemagglutination Activity Relationships; Uncentrifuged Virus Suspensions.

Sample No.	Particles/ml ($\times 10^{10}$)	PFU/ml ($\times 10^8$)	HA units/ml ($\times 10^2$)	Particles/PFU	Particles/HA unit ($\times 10^7$)	PFU/HA unit ($\times 10^5$)
1-4	4.1	2.3	8	180	5	3
2-5	4.7	3.3	9.6	140	5	3
3-6	5.3	3.8	8	140	7	5
4-8	4.8	1.4	4	340	12	4
Avg	—	—	—	200	7	4

TABLE IV. Particle Count—Infectivity—Hemagglutination Activity Relationships; Supernatant Virus after Low Speed Centrifugation.

Sample No.	Particles/ml ($\times 10^9$)	PFU/ml ($\times 10^7$)	HA units/ml ($\times 10^2$)	Particles/PFU	Particles/HA unit ($\times 10^6$)	PFU/HA unit ($\times 10^4$)
1- 7	1.1	2.0	1.6	55	7	12
2- 8	2.4	6.4	8.0	38	3	8
3-10	3.6	12	8.0	30	5	15
4-12	4.4	15	8.0	30	6	19
Avg	—	—	—	38	5	14

TABLE V. Particle Count — Infectivity — Hemagglutination Activity Relationships Using Whole, Uncentrifuged Virus Suspensions and Their Supernatants after Low Speed Centrifugation.

Sample No.	Preparation	Particles/ml ($\times 10^9$)	PFU/ml ($\times 10^7$)	HA units/ml ($\times 10^3$)	Particles/PFU	Particles/HA unit ($\times 10^6$)
216	Whole	63	15	3.8	420	20
	Supernatant	4.7	10	2.6	47	2
217	Whole	24	12	5.1	200	5
	Supernatant	4.1	11	5.1	37	.8

probably virus or virus related structures because we have never seen them in cultures not infected with polyoma virus.

There was a consistent correlation between particle counts and biological titrations of similar materials. Extensively aggregated, unsedimented materials were characterized by high particle:infectivity and high particle:hemagglutinating unit ratios, as would be expected. With virus suspensions from which gross aggregates were removed by low speed centrifugation, much lower ratios were obtained. Experiments are under way to find ways to de-aggregate polyoma virus without causing a loss in biological activity. Apparently large aggregates play no detectable role in the infectivity and hemagglutination activity of polyoma virus suspensions although as much as 92% of the virus may be in this form. Since infectivity measurements do not detect 90% of virus mass in such preparations, particle counting is probably a more dependable measure of total virus production.

Efforts are being made to increase the sensitivity of particle counting so that dependable counts may be made when the virus is in very low concentrations relative to cell debris. We are continuing the study of this virus-cell relationship with both biological and physical methods in the hope that new information can be obtained.

Summary. A method is described for quantitative counting of polyoma virus particles with a precision of about 16%. The method involves sedimentation of virus upon agar in the swinging-bucket ultracentrifuge rotor, pseudoreplication, and electron microscopy of the particles. The particle counts were correlated quantitatively with biological activity. Aggregation is an outstanding feature of this strain of polyoma virus. When this was minimized, a particle count to infectivity ratio of 38:1 was obtained. Approximately 5×10^6 particles were equivalent to 1 hemagglutinating unit.

1. Dulbecco, R., Freeman, G., *Virology,* 1959, v8, 396.

2. Vogt, M., Dulbecco, R., *Proc. Nat. Acad. Sci.,* 1960, v46, 365.

3. Sharp, D. G., Proc. 4th Intern. Congr. Electron Microscopy, Berlin, 1958, 542 (published, 1960).

4. Smith, K. O., Sharp, D. G., *Virology,* 1960, v11, 519.

5. Howatson, A. F., Almeida, J. D., *J. Biophys. Biochem. Cytol.,* 1960, v8, 828.

Received April 21, 1961. P.S.E.B.M., 1961, v107.

Modification of Irradiation Effects in the Pigeon, *Columba livia*. II. Effects of Delayed Bone Marrow Implantation.* (26643)

Don H. Shaw and Halvor Vermund
Departments of Radiology and Genetics, University of Wisconsin, Madison

There is general agreement that grafts of autologous, isologous, homologus and, in most instances, heterologous bone marrow prolong the life of lethally irradiated animals, by preventing death due to damage of the hematopoietic system(1). Marrow transplantation has been of limited therapeutic value, because the majority of irradiated individuals grafted with homologous or heterologous marrow ultimately die from a wasting disease ("homologous or heterologous disease")(2).

Shaw *et al.*(3,4,5), using the heterotransplant system of pigeons and doves, demonstrated that both host-species-specific hemagglutinins and precipitins were detectable in the circulation of pigeon-dove chimeras implanted *immediately* after irradiation. An immunologic response of the implanted marrow directed against the host tissue was suggested in line with the general theories proposed by Ford *et al.*(6).

The lack of success from homologous marrow implantation in irradiated humans(7) may be related to similar mechanisms or to an immunologic response of the regenerated host tissues directed against the antigenically foreign marrow implant(8).

Mathé *et al.*(9,10) reported remarkable success, however, in 4 of 5 Yugoslavians accidentally exposed to large doses of irradiation, with homologous marrow injected 30 days after the irradiation. In each of these cases partial repopulation by the donor erythrocytes occurred followed by chimera reversal (*i.e.*, the host hematopoietic system recovered and replaced the implanted marrow system).

The present report includes findings of experiments in which "immediate" and "delayed" (5 to 7 days post-irradiation) implantation of homologous or heterologous bone marrow was employed in pigeons subjected to lethal total body irradiation.

Material and methods. Dose response curves were established and showed that the effective LD 100/30 for the pigeon (*Columba livia*) was 2,500 rads. The source of the birds, methods of irradiation, marrow preparation and injection were as previously described(3).

A total of 241 pigeons were used: 211 received 2,500 rads total-body and 30 were maintained as unirradiated controls. The birds were separated into the following groups:

Group 1: *Immediate homotransplants*: 40 irradiated birds given single intravenous injection of 2 ml of a saline suspension of fresh homologous marrow within 4 to 6 hours after irradiation.

Group 2: *Immediate heterotransplants*: 40 irradiated birds injected intravenously with 2 ml of a saline suspension of fresh heterologous marrow from *Streptopelia risoria* within 4 to 6 hours after irradiation.

Group 3: *Delayed homotransplants*: 50 irradiated pigeons, 30 treated June 1960 (Series A) with 250×10^6 nucleated homologous marrow cells in groups of 10 on days 5, 6 and 7 postirradiation respectively; 20 treated Aug. 1960 (Series B) with the same type and quantity of marrow cells but all on the 5th day after irradiation.

Group 4: *Delayed heterotransplants*: 50 irradiated pigeons treated in the same way as Group 3 except that heterologous dove marrow was injected.

Group 5: *Irradiated controls*: 31 birds injected with 2 ml of sterile saline intrave-

* From the Dept. of Radiology, Medical School, and the Dept. of Genetics (paper No. 837) Agr. Exp. Station, Univ. of Wisconsin. This project was supported in part by the Research Committee of the Graduate School from funds supplied by Wisconsin Alumni Research Foundation and in part by grants to M. R. Irwin and to Halvor Vermund from Dept. of HEW, Nat. Inst. Health, P.H.S.

nously; 10 at the same time as birds in Groups 1 and 2, randomized and housed in the same cages, 11 with Groups 3 and 4, Series A, and 10 with Groups 3 and 4, Series B.

Group 6: *Unirradiated birds*: 30 pigeons injected as in Group 5, 10 with Groups 1 and 2 and 10 each for Series A and B.

The homologous bone marrow was obtained from pigeons whose erythrocytes were distinguishable from those of the host by one or more of the erythrocytic antigenic markers B^g, C^g, and E^g derived from *C. guinea* which were previously designated as B, C and E(11). In certain donor-host combinations the erythrocytes differed in their reaction with the seed extracts of Sieva (*Phaseolus limensis*)(3) and *Bandeiraea simplicifolia*. Each of these 2 phytoagglutinins distinguished between the erythrocytes of individual pigeons; *i.e.*, those whose red cells were agglutinated were designated as Si^+ and Ba^+, respectively, while those with non-reactive red cells were Si^- and Ba^-. Since these erythrocytic properties appeared to be genetically determined and were autonomous (La Bar and Shaw, unpublished), they were used as blood group markers in the same manner as the above mentioned erythrocytic antigenic markers.

Blood samples were drawn for both sera and erythrocytes from the wing veins of all birds on days 0, 4, 6, 8, 10, 12, 20, 28, 30, 35, 40 and 45, or until the birds died. The samples of erythrocytes from Groups 1, 3-A and 3-B were tested with antisera specific for the B^g, C^g and E^g antigenic factors, to reveal the presence or absence of the donor's erythrocytes. They were also tested with saline extracts of the seeds of the Sieva and *Bandeiraea simplicifolia* as well as with antisera specific for the B^l antigenic factor of *C. livia*, previously called B′(11), to detect the host's or donor's red cells.

Samples of cells from Groups 2, 4-A and 4-B were tested with rabbit anti-*livia* serum (absorbed with cells of *risoria*, rendering it specific for *livia* erythrocytes in contrast to those of *risoria*) to detect pigeon (host) red cells. These samples were also tested for the presence or absence of red cells derived from the bone marrow of the donor with an antiserum (rabbit anti-*risoria* serum absorbed with cells of *livia*) which was reactive with *risoria* erythrocytes, but not with those of *livia*.

The serum samples from birds in Groups 1-6 were tested for possible hemagglutinins against a panel of erythrocyte suspensions from *livia*, *risoria*, and backcross hybrids carrying the B^g, C^g and E^g factors from *Columba guinea*. In addition, each sample of serum from each bird was tested against its own erythrocytes (obtained shortly before the implantation), and against cells of the bone marrow donor to determine if there were hemagglutinins present which were reactive with the erythrocytes of either or both the host or donor prior to implantation. The sera obtained from the heterotransplant groups do exhibit hemagglutinin activity at various times (Table III).

Since antibiotic therapy has been successful in eliminating early deaths, presumably due to bacterial invasion *via* the radiation damaged intestinal tract in both rats(12) and rabbits(13) given lethal total body irradiation, it was employed in these pigeons to reduce early deaths (within 3-5 days). The birds in this study were given Myzon Poultry Builder (terramycin plus vitamins and minerals) in their drinking water starting 10 days prior to irradiation and were maintained on this antibiotic throughout the experimental period. Concentration of the active component was .05 g/liter of Oxytetracycline hydrochloride (Terramycin R).

Results. The data in Table I clearly indicate that survival was increased for both homologous and heterologous bone marrow implants when the implant was delayed 5 to 7 days after irradiation. For the *immediate transplant* groups average survival time was 24.2 days after homologous and only 8.8 days after heterologous bone marrow implantation. In contrast, for the *delayed transplant* average survival time was 46.6 days after homologous and 26.9 days after heterologous implants. *Delayed* bone marrow transplantation in contrast to *immediate* transplantation, resulted in a highly significant increased average survival time from

TABLE I. Survival of Lethally Irradiated Pigeons Receiving Immediate or Delayed Homo- or Heterotransplants.

Treatment		Total No.	No. of survivors Days postirradiation									Avg survival
Type	Day	treated	4	6	10	14	18	30	45	60	90	time, days
HBM (Group 1)	0	40	38	38	23	20	15	9	4	3	2	24.2 ± 3.6
DBM (Group 2)	0	40	34	26	10	6	3	0	—	—	—	8.8 ± .6
HBM (Group 3)	5	10	—	10	9	8	6	4	4	3	2	49.8
Series A	6	10	—	10	8	7	6	3	2	2	2	44.1
	7	10	—	10*	9	7	7	2	2	1	1	42.3
Series B	5	20	—	20	18	15	13	7	6	4	4	47.8
Delay total	—	50	—	50	44	37	32	16	14	10	9	46.6 ± 2.2
DBM (Group 4)	5	10	—	10	9	4	2	2	2	2	1	37.7
Series A	6	10	—	10	8	4	1	0	—	—	—	19.4
	7	10	—	10*	8	6	3	1	1	1	1	27.2
Series B	5	20	—	20	17	10	4	2	2	1	1	25.7
Delay total	—	50	—	50	42	24	10	5	5	4	3	26.9 ± 5.0
Irrad. controls	—	31	31	29	17	11	4	0	—	—	—	13.3 ± 1.0
Unirrad. controls	—	30	30	30	30	30	30	30	30	30	30	

* Day 7.

HBM = Homologous bone marrow, from another pigeon. DBM = Dove (heterologous) bone marrow.

Day 0 = time of transplant after irradiation (approx. 4 to 6 hr). Day 5, 6, or 7 = No. of days between irradiation and time of transplant.

24.2 ± 3.6 to 46.6 ± 2.2 days ($p = .0002$) for homotransplants, and from 8.8 ± 0.6 to 26.9 ± 5.0 days ($p = .0003$) for heterologous marrow implants. There was no significant difference in average survival between the 3 delayed implant groups, although the homologous transplant data suggested that day 5 might be therapeutically somewhat superior to day 7. To answer this question adequately, a larger number of birds would be necessary and an extended time range used.

Of particular interest was the fact that 3 of the pigeons with the *delayed heterotransplants* (Group 4-A) showed prolonged survival time: to the 66th, 126th and 186th day respectively, the 2 latter being shown in Table III (No. 3106.34 and No. 3106.38). This in contrast to a maximum of 19 days survival time for the *immediate heterotransplants* (Group 2), indicated a substantial increase in survival time when the heterotransplant was delayed.

Table II shows the immunologic data on erythrocytic repopulation, which demonstrated that in the *delayed homotransplants* (Groups 3-A and 3-B) which survived to the 60th day, there was only partial donor repopulation and that chimera reversal occurred (see bird No. 3106.6) or at least a proportion of donor cells in circulation reached a maximum and then declined (see bird No. 3106.63). This was not the result previously reported(3), and differed also from the data for birds in the *immediate transplant* Group 1 of this present report. In these latter 2 cases partial and eventual total repopulation of the erythrocytes resulted (see birds Nos. 3100.33 and 3100.44).

Table III contains the immunologic findings for the *delayed* and *immediate heterotransplants* (Groups 2, 4-A and 4-B). It is interesting to note that, whether the dove marrow was implanted immediately or on days 5, 6 or 7, "*in vivo* agglutination" occurred between the 4th and 6th day after such a heterotransplant was made, regardless of the time elapsed after irradiation. As indicated in Table III, when dove marrow was transplanted immediately (Group 2), host-species-specific hemagglutinins appeared in the circulation of the irradiated host and were sufficiently potent to produce a high degree of "*in vivo* agglutination" (titer with

TABLE II. Representative Immunologic Data of Results Obtained in Erythrocyte Repopulation Studies of Irradiated Pigeons Injected with Homologous Bone Marrow on Day 0, 5, 6 and 7.

Bird No.	Blood type of donor	Reagents used to test RBC	Agglutination reactions of erythrocytes* Days postirradiation 0	8	14	20	28	45	60	70	90	Survival time (days)
3100.24	B^g	Sieva	4+	4+								9
Day-0	Si^-	Anti-B^g	0	0								
		Anti-C^g	0	0								
3100.13	B^g & C^g	Sieva	4+	4+	2+	1+	0					30
Day-0	Si^-	Anti-B^g	0	0	2+	3+	3+					
		Anti-C^g	0	0	3+	3+	4+					
3100.33	B^g & C^g	Sieva	4+	3+	2+	1+						21
Day-0	Si^-	Anti-B^g	0	0	2+	3+						
		Anti-C^g	0	±	3+	3+						
3100.44	B^g & C^g	Sieva	3+	3+	2+	1+	0	0	0	0		78
Day-0	Si^-	Anti-B^g	0	0	2+	2+	3+	4+	4+	4+		
		Anti-C^g	0	0	2+	3+	3+	4+	4+	4+		
3106.5	B^g & C^g	Ba-Ext.	0	0	0	1+	2+	±	0	0	0	131
Day-6	Ba^-	Anti-B^g	0	0	0	2+	3+	2+	1+	0	0	
		Anti-C^g	0	0	±	2+	3+	2+	1+	0	0	
3106.63	B^gB^g	Ba-Ext.	0	0	0	0	0	0	0	0	0	124
Day-5	Ba^-	Anti-B^g	0	0	±	2+	3+	2+	2+	1+	± 1+	
		Anti-B^1	4+	4+	3+	3+	3+	3+	3+	3+	3+	
3106.62	B^gB^g	Ba-Ext.	2+	2+	2+	1+						26
Day-5		Anti-B^g	0	0	2+	3+						
		Anti-B^1	4+	4+	3+	1+						
3106.69	B^g	Ba-Ext.	3+	3+								12
Day-5	Ba^-	Anti-B^g	0	0								
		Anti-C^g	0	0								

* Degrees of agglutination were as follows: Complete (4+); approx. 75% (3+); approx. 50% (2+); approx. 25% (1+); detectable but less than 20% (±); and not detectable (0).

pigeon cells ranging from 8 to 512) and persisted until death. The few birds which survived beyond the 10th day showed complete erythrocytic repopulation by the marrow implant (see bird No. 3100.37). This is in complete accord with previous findings(3,4). Total erythrocytic repopulation was not evident when delayed marrow implants were made (Group 4-A and B). Although the host-species-specific hemagglutinins occurred in circulation between the 4th and 6th days after the implantation their titer was somewhat lower (titer with pigeon cells ranging 8 to 64) than those observed in the *immediate heterotransplants* yet sufficiently potent to produce "*in vivo* agglutination," but without all the host's erythrocytes being eliminated. For a period both the host's and the donor's erythrocytes were present in the circulation. Approximately 15 days after implantation, or 20 days after irradiation, the host-species-specific hemagglutinins disappeared. By the 30th day after implantation (35 days after irradiation) donor-species-specific hemagglutinins appeared in the circulation and, although these agglutinins ranged in titer from 8 to 16 with dove cells, no "*in vivo* agglutination" was observed. This was possibly due to the relatively small number of erythrocytes in the circulation derived from the implanted dove marrow. On the 45th day postirradiation chimera reversal was completed, *i.e.*, the only erythrocytes detectable by the agglutination procedure were of the host's (pigeon) specificity (see birds Nos. 3106.34 and 3106.38).

Due to the high radiation tolerance of pigeons (LD 100/30 = 2,500 rads), damage to the intestinal mucosa might be expected to be more severe than in mice. This was illustrated by the relatively large percentage of early deaths (within 3 to 5 days), presumably due to bacterial invasion, obtained in previous experiments(3). Pilot experiments with antibiotic therapy in pigeons have indicated that an increase in survival oc-

TABLE III.* Immunologic Data for 5 of the Irradiated Pigeons Injected with Heterologous Bone Marrow, Showing Each of the Different Types of Results Obtained.

Bird No.	Types of observations	Time blood samples were obtained (days postirradiation) 0	4	6	10	12	20	35	45	Survival time
3100.16	Degree of "*in vivo* agglutination"	0	2+	3+						6
Day-0	Hemagglutinin activity of serum with RBC's of: *livia*	0	2+	3+						
	risoria	0	0	0						
3100.37	Degree of "*in vivo* agglutination"	0	1+	3+	0	0				19
Day-0	Hemagglutinin activity of serum with RBC's of: *livia*	0	1+	3+	4+	4+				
	risoria	0	0	0	0	0				
	RBC activity with: Anti-*livia*	4+	4+	4+	† 0	0				
	Anti-*risoria*	0	1+	3+	4+	4+				
	Isotonic NaCl	0	1+	3+	0	0				
3106.34	Degree of "*in vivo* agglutination"	0	nt	0	1+	2+	1+	0	0	126
Day-6	Hemagglutinin activity of serum with RBC's of: *livia*	0	nt	0	±	3+	3+	0	0	
	risoria	0	nt	0	0	0	0	2+	3+	
	RBC activity with: Anti-*livia*	4+	nt	4+	4+	3+	2+	† 4+	4+	
	Anti-*risoria*	0	nt	0	1+	2+	2+	0	0	
	Isotonic NaCl	0	nt	0	1+	2+	1+	0	0	
3106.38	Degree of "*in vivo* agglutination"	0	nt	0	1+	2+	1+	0	0	186
Day-5	Hemagglutinin activity of serum with RBC's of: *livia*	0	nt	0	2+	3+	2+	0	0	
	risoria	0	nt	0	0	0	0	3+	2+	
	RBC activity with: Anti-*livia*	4+	nt	4+	4+	3+	2+	† 4+	4+	
	Anti-*risoria*	0	nt	0	2+	2+	3+	±	0	
	Isotonic NaCl	0	nt	0	1+	2+	1+	0	0	
3106.68	Degree of "*in vivo* agglutination"	0	nt	0	2+	3+				14
Day-5	Hemagglutinin activity of serum with RBC's of: *livia*	0	nt	0	2+	3+				
	risoria	0	nt	0	0	0				
	RBC activity with: Anti-*livia*	4+	nt	4+	4+	4+	± †			
	Anti-*risoria*	0	nt	0	1+	3+				
	Isotonic NaCl	0	nt	0	1+	3+				

* Symbols for degrees of agglutination same as used in Table II.

† Agglutination in these samples is due to *in vivo* agglutination though it appears that specific antisera increase degree of agglutination.

curred in all irradiated groups (both transplanted and controls) and deaths prior to day 5 were eliminated. Additional studies along this line suggested that even more effective protection against early death was obtained when antibiotic therapy was started 10 days prior to irradiation. The therapeutic effect was not, however, sufficient to give 30 day survival in either the *immediate heterotransplant* or in the irradiated control groups.

Discussion. It appears probable from these data that delaying the implant for a period of 5 to 7 days allowed time for the irradiated host's hematopoietic system to recover (*i.e.*, a portion of the host's hematopoietic cells, though probably in a state of mitotic arrest, was not destroyed by the irradiation and had had time to recover). This would not be the case, however, when immunologically antagonistic donor marrow was implanted immediately. Under these conditions, if the implanted marrow was immunologically competent, it would be expected that the donor's immunologic mechanism would destroy the residual host tissue before recovery was possible. This certainly appeared to be true in the pigeon studies as borne out by the "*in vivo* agglutination"

which occurred in *immediate heterotransplant* pigeon-dove chimeras, apparently resulting from the host-species-specific hemagglutinins present. As previously reported(3, 4), the most plausible interpretation for the existence of these hemagglutinins is that they were elaborated by, or their specificity was directed by, the cells of the heterotransplants. Other reports(14,15,16) also indicate immunologic competence of the donor marrow.

The sequence of changes described suggests that the donor's bone marrow might coexist with the residual hematopoietic tissues of the host, each exerting its own immunologic influence. Furthermore, the evidence suggests that the host tissues might have a selective advantage which tends to result in chimera reversal. Under the conditions which appeared to exist in the *delayed implant* groups, the chances of an acute immunologic reaction sufficient to result in death were reduced. The data in this report, along with information on the Yugoslavians exposed to iradiation by accident and treated with homologous marrow(9,10), indicated that the best therapeutic effects may be achieved if the host's and donor's immunologic responses are modified during a critical period after the implantation period. Such modifications, properly manipulated, could result in chimera reversal so that the final result would be an individual with its own hematopoietic and immunologic systems restored. The donor marrow under these conditions would serve only to supply necessary formed elements and other substances during a period when the host's system is inadequate due to radiation injury. Under certain conditions a balanced chimera would also be expected to survive, *i.e.,* if actively acquired tolerance developed in both the host and the grafted tissue. Chimera reversal or balanced chimerism appears indeed essential for prolonged survival of lethally irradiated animals grafted with "foreign" marrow. Predictability and some degree of control of such reversal by adjusting size of inoculum and time of administration would be a major step toward therapeutic utilization of bone marrow implantation.

Summary. 1. *Delayed* bone marrow *transplantation,* in contrast to *immediate transplantation,* resulted in an increased average survival time of approximately 20 days for both homologous and heterologous marrow in lethally irradiated pigeons. 2. *Delayed homotransplantation* resulted in only partial repopulation by donor derived erythrocytes. Chimera reversal occurred or the proportion of donor erythrocytes in circulation reached a maximum and then declined, while *immediate homotransplantations* resulted in eventual total repopulation by donor erythrocytes. 3. In the heterologously treated pigeons "*in vivo* agglutination" occurred between the 4th and 6th day *after heterotransplantation* regardless of the number of days after irradiation. 4. *Delayed heterotransplantation* did not result in elimination of all the host's ertyhrocytes, as was the case for the *immediate heterotransplants.* However, host-species-specific hemagglutinins were detectable at titers ranging from 8 to 64 with host cells, and sufficiently potent to produce "*in vivo* agglutination". 5. The host-species-specific hemagglutinins, which were present in the *delayed heterotransplants,* disappeared 15 to 20 days after the marrow implant and by the 30th day donor-species-specific hemagglutinins appeared in the circulation. In contrast, the host-species-specific hemagglutinins persisted until death (longest survivor, 19 days) in the *immediate heterotransplants.* 6. Chimera reversal occurred in all 5 of the *delayed heterotransplants* which survived until the 45th day postirradiation. In contrast, the longest survival time after *immediate heterotransplantation* was 19 days and total repopulation by donor erythrocytes occurred in all animals which survived 10 days or longer.

1. Congdon, C. C., Uphoff, D., Lorens, E., *J. Nat. Cancer Inst.,* 1952, v13, 73.

2. Trentin, J. H., *Ann. N. Y. Acad. Sci.,* 1958, v73, 799.

3. Shaw, D. H., Vermund, H., *Proc. Nat. Acad. Sci.,* 1959, v45, 23.

4. ———, *Radiation Res.,* 1959, v11, 466.

5. Shaw, D. H., Mueller, M. J., LaBar, M. M., *Fed. Am. Soc. Exp. Biol.,* April 1960, in press.

6. Ford, C. E., Hammerton, J. L., Barnes, D. W. H., Loutit, J. F., *Nature,* 1956, v177, 452.

7. Thomas, E. D., Lochte, H. H., Jr., Ferrebee, J. W., *Blood, J. Hemat.*, 1959, v14, 1.

8. Makinodan, T., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 174.

9. Mathé, G., Jammet, H., Pendic, B., Schwarzenberg, L., Duplan, J. F., Maupin, B., Latarjet, R., Larrieu, M. J., Kalic, D., Djukic, J., *Clin et Biol.*, 1959, v4, 226.

10. Jammet, H., Mathé G., Pendic, B., Duplan, J. F., Maupin, B., Latarjet, R., Kalic, D., Schwarzenberg, L., Djukic, Z., Vigne, J., *ibid.*, 1959, v4, 210.

11. Miller, W. J., Bryan, C. R., *Proc. Nat. Acad. Sci.*, 1956, v39, 174.

12. Takota, T. S., *Radiation Res.*, 1959, v11, 471.

13. Porter, K. A., Murray, J. E., *J. Nat. Canc. Inst.*, 1958, v20, 189.

14. Trentin, J. J., *Ann. N. Y. Acad. Sci.*, 1958, v73, 799.

15. Feldman, M., Yaffe, D., *J. Nat. Canc. Inst.*, 1958, v21, 697.

16. Upheff, D. E., Law, L. W., *ibid.*, 1959, v22, 229.

Received April 24, 1961. P.S.E.B.M., 1961, v107.

Some Pharmacologic Properties of Pangamic Acid (Vitamin B-15).* (26644)

F. N. MARSHALL, R. H. ADAMSON AND J. P. LONG

Department of Pharmacology, College of Medicine, State University of Iowa, Iowa City.

Pangamic acid (dimethyl-amino-acetyl-gluconic acid) also known as Vit. B-15, was discovered by E. T. Krebs in 1938. The presence of this vitamin has been demonstrated in apricot kernels, rice bran, brewer's yeast, ox blood and horse liver. It is thought to accompany the other members of the Vit. B complex(1,2). Although pangamic acid has been reported to be without pharmacologic properties, it has been suggested for use in treatment of cardiovascular and rheumatic diseases, alcoholism and liver cirrhosis(1, 2,3). This investigation deals with some pharmacologic properties of pangamic acid. Although this compound appears to be structurally dissimilar from thiamine, this investigation demonstrates that pangamic acid shares some of the pharmacologic actions of thiamine(4,5,6).

Methods. Neuromuscular blocking activity was evaluated by using the sciatic nerve-gastrocnemius muscle preparation in the rabbit and chicken. Mature, Dutch rabbits were anesthetized with sodium phenobarbital (200 mg/kg) and bipolar, silver electrodes were placed on the peripheral end of the sectioned sciatic nerve. The nerve was stimulated for 0.2 second with monophasic, square wave pulses of 2 milliseconds duration at a frequency of 250 per second and of supramaximal voltage delivered every 5 seconds. A Grass stimulator (Model S4C) with circuit interrupter was used in all experiments. Semi-isometric recordings were made on smoked kymograph paper. Sixteen animals were used to evaluate the activity of pangamic acid. A linear regression and the dose producing 50% neuromuscular blockade (ED-50) and its 95% confidence interval were calculated as outlined by Finney(7). The neuromuscular effects of pangamic acid were also studied in the chicken using the method described by Pelikan *et al.*(8). The parameters described for the rabbit sciatic nerve-gastrocnemius muscle preparation were also used in this preparation.

The effect of pangamic acid on blood pressure was studied in 20 anesthetized dogs (thiopental soduim 15 mg/kg and barbital sodium 250 mg/kg). Carotid or femoral arterial pressure, electroencephalographic (EEG) and electrocardiographic (ECG) recordings were made. In 5 dogs, the left fore limb was perfused with blood from the abdominal aorta using a constant flow Sigmamotor pump. Injections were made into the perfusing blood or given systemically. Systemic arterial blood pressure and fore limb perfusion pressure were recorded simultaneously. Drug action on the isolated rabbit heart was studied by the clas-

* This investigation supported in part by USPHS Training Grants.

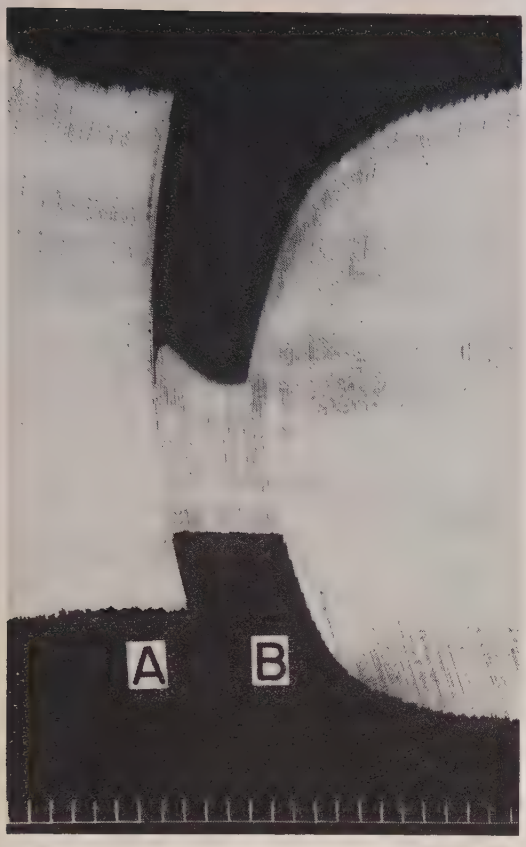


FIG. 1. Effect of neostigmine methylsulfate upon neuromuscular blockade produced by pangamic acid. Time marks equal 1 min. A, pangamic acid, 100 mg/kg. B, neostigmine methylsulfate, 50 μg/kg.

sical method of Langendorf(9). The effect of pangamic acid on spontaneous contractions of isolated rabbit ileum was also studied. Solutions of pangamic acid (Nutritional Biochemicals) were freshly prepared and administered intravenously in the neuromuscular and blood pressure studies.

Intravenous and intraperitoneal LD-50 values were obtained by determining the effect of at least 3 dose levels yielding between 10 and 90% effect. At least 10 mice (Rockland strain, Sutter Farms) were utilized in determining each point. Computations for fitting the probit-log dose regression lines determining the LD-50 and 95% confidence intervals were done according to the method of Finney(7).

Results and discussion. Toxicity studies in mice showed that the LD-50 for pangamic acid following intraperitoneal administration was 1349 mg/kg with 95% confidence limits of 1148 to 1514 mg/kg. This value is considerably lower than that reported by Krebs *et al.*(1) and Millet(2): 14,700 ± 650 mg/kg. The LD-50 value following intravenous administration was found to be 255 mg/kg with 95% confidence limits of 170 to 513 mg/kg. The mice died of respiratory paralysis as evidenced by the fact that upon opening the chest cavity the heart continued to beat.

The dose of pangamic acid causing 50% neuromuscular blockade in rabbits was 129 mg/kg with 95% confidence limits of 126 to 135 mg/kg. The blockade was rapid (1-2 min) in onset and reversible; the duration of blockade being dose related. Neuromuscular blockades of 70-80% returned to control levels within 2 hours. Neostigmine methylsulfate was found to be an effective antagonist of the blockade (Fig. 1). In the chicken, pangamic acid produced a flaccid paralysis which suggests a non-depolarizing type of blockade.

Intravenous administration of pangamic acid in anesthetized dogs resulted in a rapid depressor response of variable duration. A dose of 5 mg/kg (8 dogs) produced a fall in blood pressure of 24 ± 7 mm of mercury. A slight sinus bradycardia was the only observable change found in the ECG. Blood pressure responses of small doses of epinephrine, levarteranol, acetylcholine, histamine and tetramethyl ammonium measured before and after administration of pangamic acid did not differ markedly. The pressor response to bilateral carotid occlusion before and after drug was not altered. Neither vagal section nor administration of atropine sulfate (1 mg/kg), scopolamine hydrobromide (1 mg/kg), chlorprophenpyridamine maleate (25 mg/kg) nor promethazine hydrochloride (25 mg/kg) altered the magnitude of the depressor response produced by pangamic acid.

Fore limb perfusion studies indicated that peripheral vasodilatation does not contribute markedly to the depressor response seen when pangamic acid is administered intravenously.

In the isolated rabbit heart, pangamic acid

in doses up to 20 mg dissolved in Locke-Ringer solution and injected into the coronary inflow cannula produced only slight, transient depression of contractions and some reduction of rate.

Concentrations of pangamic acid up to 6 $\times 10^{-4}$ M did not alter spontaneous contractions of the isolated rabbit ileum, nor did this concentration of drug modify contractions produced by acetylcholine.

Our data demonstrate that pangamic acid is not without pharmacologic effect. We have found the LD-50 in mice after intraperitoneal administration to be much lower than the value previously reported for this compound. It has been reported that thiamine hydrochloride possesses neuromuscular blocking activity and that this effect is antagonized by neostigmine methylsulfate(4,5, 6). It has also been demonstrated that intravenous administration of thiamine hydrochloride produced a transient but marked hypotension with concomitant bradycardia in anesthetized dogs. In these respects, pangamic acid produces pharmacological effects not unlike those of thiamine hydrochloride. Circulatory collapse has been reported clinically from intravenous and intramuscular injections of thiamine hydrochloride(10). We are not aware of any clinical reports of respiratory depression or severe hypotension resulting from administration of pangamic acid. However, it is conceivable that pangamic acid could produce undesirable clinical effects under circumstances of overdosage.

Summary. Pangamic acid (Vit. B-15) possesses definite pharmacologic properties. These include neuromuscular blocking activity in the rabbit and chicken as well as production of hypotension in anesthetized dogs. Neostigmine methylsulfate is an effective antagonist to the neuromuscular blockade produced in the rabbit. These effects appear to be qualitatively similar to those reported for thiamine hydrochloride.

1. Krebs, E. T., Sr., Krebs, E. T., Jr., Beard, H. H., Malin, R., Harris, A. T., Barlett, C. L., *Intern. Record of Med.,* 1951, v164, 18.

2. Millet, R. C., *Rev. Clin. Espanola,* 1957, v64, 196.

3. Dettemante, M., *Farm. y terap.* (Madrid), 1958, v64, 145.

4. Pick, E. P., Unna, K., *J. Pharmacol. Exp. Therap.,* 1945, v83, 59.

5. Smith, J. A., Foa, P. P., Weinstein, H. R., Ludwig, A. S., Wertheim, J. M., *ibid.,* 1948, v93, 294.

6. ———, *Science,* 1948, v108, 412.

7. Finney, D. J., Statistical Method in Biological Assay, Hafner Pub. Co., New York, 1952.

8. Pelikan, E. W., Smith, C. M., Unna, K. R., *J. Pharmacol. Exp. Therap.,* 1954, v111, 30.

9. Langendorf, O., *Pfluger's Arch. f. ges. Physiol.,* 1895, v61, 291.

10. Chitwood, W. R., Moore, C. D., *J.A.M.A.,* 1952, v148, 461.

Received April 3, 1961. P.S.E.B.M., 1961, v107.

Leukokinetic Studies V. Uptake of Tritiated Diisopropylfluorophosphate by Leukocytes.* (26645)

DORIS KURTH, J. W. ATHENS, E. P. CRONKITE, G. E. CARTWRIGHT AND M. M. WINTROBE

Department of Medicine, University of Utah College of Medicine, Salt Lake City, and Medical Research Center, Brookhaven National Laboratory, Upton, N. Y.

The use of radioactive diisopropylfluorophosphate (DFP^{32}) as a label for leukocytes has been described in previous reports from this laboratory(1-3). Leukocytes have been labeled *in vitro* and returned to the circulation of the donor(2), or they have been labeled *in vivo* by administering the DFP^{32} intravenously(1,3). During these studies it was demonstrated that polymorphonuclear (PMN) neutrophils but not lymphocytes are

* This investigation was supported in part by a research grant from Nat. Inst. of Arthritis and Metab. Dis., Nat. Inst. Health, U.S.P.H.S. and in part by U. S. Atomic Energy Commission.

labeled by DFP^{32}. It was not possible to determine whether eosinophils, basophils and monocytes are labeled.

When leukocytes were labeled *in vivo* following intravenous administration of DFP^{32}, the blood leukocyte radioactivity curve obtained was complex(1,3). The shape of the curve suggested that granulocytes in the bone marrow as well as in the circulation were labeled, but that the circulating granulocytes, due to exposure to a higher initial concentration of DFP^{32}, were labeled about 3 times more heavily than those in the bone marrow.

The purpose of the present communication is to present data on uptake of tritiated DFP (H^3-DFP) by various morphologic types of leukocytes under both *in vitro* and *in vivo* labeling conditions. The binding of H^3-DFP by leukocytes obtained from both normal and leukemic subjects has been investigated and the relative degree of labeling of both blood and marrow leukocytes has been determined following intravenous administration of H^3-DFP.

Materials and methods. Tritiated DFP (H^3-DFP) was purchased as a solution in anhydrous propylene glycol from New England Nuclear Co., Boston, Mass. By special arrangement high specific activity H^3-DFP (2.2 mc/mg) was synthesized from isopropyl-2-H^3 alcohol. The DFP content of the material was measured within a week before use by the colorimetric method of Marsh and Neale(4).

Blood was labeled *in vitro* in 2 different ways. In the first, venous blood was drawn into a plastic bag containing ACD-A (Pliapak, Abbott Laboratories) and sufficient H^3-DFP was injected into the bag to give a final concentration of 0.3 μg DFP/ml of blood. In the second method, 4 volumes of venous blood were added to siliconized test tubes (Dow Corning Z-4141) containing one volume of solution consisting of dextran, 5.0%; glucose, 2.5%; disodium ethylenediamine tetraacetate, 0.5%; sodium chloride, 0.7%; and H^3-DFP to give a final concentration of 0.3 μg/ml. In both methods the blood was mixed and incubated for one hour at room temperature. An aliquot of the leukocyte-rich supernate was removed and centrifuged at 230 g for 10 minutes. The supernate was discarded and the cells were washed once in 3 ml of the individual's own plasma. The cell button was then resuspended in 0.5 ml of plasma and thin smears were prepared on #1 coverslips. The results obtained with both labeling methods were identical.

Leukocytes were labeled *in vivo* by injecting 2 mg of H^3-DFP diluted in 20 ml of isotonic saline intravenously. Simultaneous samples of blood and marrow were obtained 1 and 3 hours later and direct smears were made on #1 coverslips.

The blood and marrow smears were fixed in methanol, mounted on glass slides and covered with AR-10 stripping film (Kodak Ltd, London). Autoradiographs were prepared according to the technic of Pelc(5) as modified by Bond *et al.*(6). The film was developed and the slides stained after time periods of from 23-70 days. The number of silver grains overlying each of approximately 1000 cells of each morphologic type, or all the cells in each smear, whichever came first, was recorded. A similar number of areas of the same size lying between the cells was examined to determine the background grain count of each preparation. Mean grain count for each cell type was calculated and mean background count for areas of corresponding size was subtracted to give mean net grain count of each cell type.

Results. Uptake of DFP by normal blood leukocytes. The distribution of silver grains in the photographic emulsion overlying lymphocytes, monocytes, eosinophils and PMN neutrophils was compared with the grains over background areas of similar size. Blood from 3 normal subjects was examined. The results obtained on one of the 3 subjects are presented in Fig. 1. Mean net grain count for each cell type in each of the 3 blood samples is given in Table I.

PMN neutrophils were labeled while eosinophils and basophils were not labeled. In one of the specimens (Fig. 1B and Table I, Subject A) the monocytes were not labeled. In the blood from the other 2 subjects (B and C) the monocytes were labeled lightly but in each specimen less than 20% of the

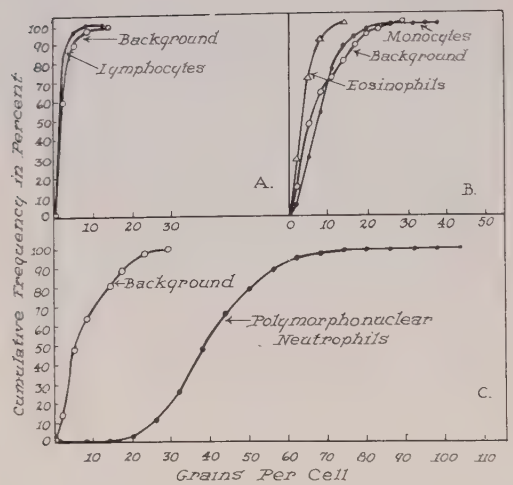


FIG. 1. Comparison of degree of labeling of normal blood leukocytes incubated *in vitro* with H^3-DFP. Distribution of grains over lymphocytes, monocytes and eosinophils is indistinguishable from background (A and B). In contrast, 85% of PMN neutrophils have more grains/cell than the most heavily labeled background areas (C).

monocytes had grain counts greater than the most heavily labeled background areas.

Typical examples of the autoradiographs obtained on normal blood are shown in Fig. 2.

Uptake of DFP by leukocytes in leukemic blood. Blood from subjects with chronic myelocytic leukemia (CML), acute myeloblastic leukemia (AML), chronic lymphocytic leukemia (CLL) and acute lymphoblastic leukemia (ALL) was incubated *in vitro* with H^3-DFP and the amount of label bound by the various morphologic cell types was determined. The results are shown in Table II.

Myeloblasts from AML and CML did not bind DFP; myelocytes contained approximately twice as much label per cell as metamyelocytes and PMN neutrophils exposed to the same concentration of H^3-DFP. Because the cytoplasmic granules were not visible in these autoradiographs it was difficult to identify promyelocytes, hence the significance of the promyelocyte grain count given in Table II may be questioned.

Lymphocytes and lymphoblasts from patients with acute or chronic leukemia did not bind DFP but those granulocytes which were present retained this property.

Since concentration of H^3-DFP and exposure time varied slightly in these studies, quantitative comparisons between samples cannot be made.

Binding of DFP by bone marrow as compared to blood leukocytes after labeling in vivo. To evaluate the initial level of cell labeling in the blood and marrow leukocytes, a hematologically normal subject with carcinoma of the mouth was given 2 mg of H^3-DFP intravenously. One and 3 hours later blood and bone marrow samples were obtained simultaneously. Autoradiographs were prepared in an identical manner so that quantitative comparison of marrow and blood labeling would be possible. The results are summarized in Fig. 3.

TABLE I. Mean Net Grain Count of Normal Blood Leukocytes Exposed to H^3-DFP.

Cell type	Subjects A	B	C
Polymorphonuclear Neutrophils	32.0	24.4	13.7
Eosinophils	−3.8	.2	.8
Lymphocytes	− .3	.5	0
Monocytes	.6	4.5	4.0

Blood from subjects A and B was labeled *in vitro*. Blood from subject C was labeled *in vivo*.

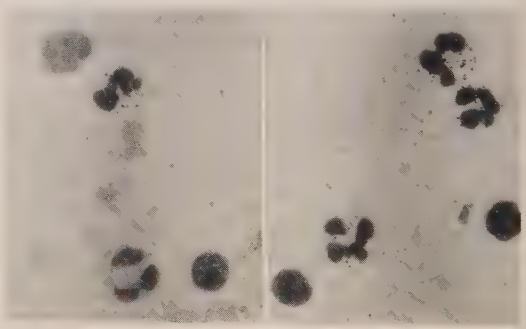


FIG. 2. Representative autoradiographs made from normal blood. Normal blood was incubated for 1 hr with 0.3 μg of H^3-DFP/ml of blood. Magnification 610 ×. (A) A monocyte and PMN neutrophil are in the left upper corner. An eosinophil and lymphocyte are in the lower right corner. Only the PMN neutrophil is labeled. (B) Three PMN neutrophils and 2 lymphocytes are seen. Again only the PMN neutrophils are labeled.

Blood PMN neutrophils contained 3 to 5 times as much label per cell as the marrow metamyelocytes and PMN neutrophils. The slightly higher grain count over the marrow PMN neutrophils as compared to the meta-

TABLE II. Mean Net Grain Count of Leukemia Blood Leukocytes Exposed to H^3-DFP.

Cell type	Subjects CML	AML	CLL	ALL
Myeloblast	.7	.1		
Promyelocyte	61.5			
Myelocyte	65.3			
Metamyelocyte	34.4			
Polymorphonuclear				
Neutrophil	27.2	28.9		29.8
Eosinophil	.2			
Basophil	.8			
Lymphoblast				.7
Lymphocyte			−.6	−.6

CML, AML, CLL and ALL refer to chronic myelocytic leukemia, acute myeloblastic leukemia, chronic lymphocytic leukemia, and acute lymphoblastic leukemia.

myelocytes was probably due to a number of more heavily labeled blood PMN neutrophils in the marrow preparations. The marked skewing of the marrow PMN neutrophil, grain-count distribution curve as compared to the other marrow granulocyte distribution curves supports this assumption.

The marrow myelocytes contained twice as much label per cell as the metamyelocytes and PMN neutrophils, a finding similar to that noted in chronic myelocytic leukemia blood (Table II).

Uptake of DFP by formed elements other than leukocytes. In the above studies label was not detectable in the erythrocytes, platelets, normoblasts or megakaryocytes.

Discussion. Myelocytes were the most heavily labeled cells in the bone marrow, while in blood only PMN neutrophils and perhaps a few monocytes were labeled.

Wachstein and Wolf(7) have demonstrated non-specific esterase in all of the formed elements of the blood and bone marrow. Furthermore, DFP^{32} has been used to label erythrocytes and platelets(8,9). However, in our studies, H^3-DFP was not demonstrated in marrow erythroblasts, myeloblasts, and megakaryocytes or in blood erythrocytes, platelets and lymphocytes. An explanation for the failure to detect H^3-DFP in these elements may be that the autoradiographic technic is relatively insensitive. It is also possible that suppression of the latent image by intracellular compounds(10) may have decreased autoradiographic efficiency over some of the cell types and thus interfered with detection of low levels of H^3-DFP binding. In any case it has been demonstrated in the present studies that concentration of DFP per granulocytic leukocyte is much greater than concentration per cell in any of the other formed elements in blood and bone marrow.

After intravenous injection of H^3-DFP, granulocytes in both the blood and bone marrow were labeled. In previous studies in which DFP^{32} was given intravenously the shape of the granulocyte disappearance curve suggested that as the heavily-labeled blood granulocytes left the circulation, they were replaced by cells from the bone marrow that contained less label. It was suggested that the bone marrow contained a store of granulocytes with about 30% as much label per cell as was initially present in the blood granulocytes(3). In an attempt to evaluate this postulate we have made quantitative estimates of degree of labeling of granulocytes in the blood and bone marrow after intravenous injection of H^3-DFP. Though we fully appreciate the difficulties in quantitative interpretations from autoradiographs, the only comparisons made here involve relative levels of labeling of different cells in the same or identically prepared autoradiographs. The present demonstration that marrow PMN neutrophils and metamyelocytes contained

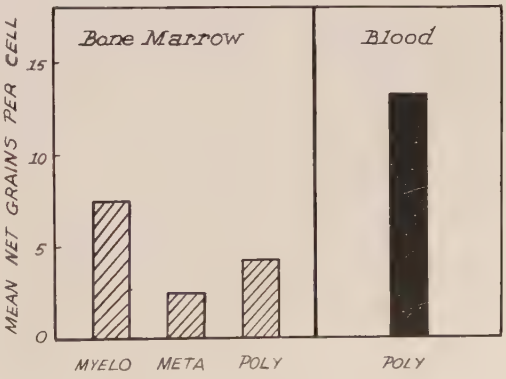


FIG. 3. Comparison of mean net grain counts in autoradiographs of marrow and blood granulocytes after intravenous injection of H^3-DFP. Mean net grain counts 3 hr after H^3-DFP injection are given.

20 to 30% as much H^3-DFP as the blood PMN neutrophils is consistent with the previous interpretation of *in vivo*, DFP^{32} granulocyte disappearance curves(3).

Conclusions. Uptake of tritiated diisopropylfluorophosphate (H^3-DFP) by various morphologic types of leukocytes after labeling *in vitro* and *in vivo* has been studied. When leukocytes from normal subjects and patients with leukemia were labeled *in vitro*, myelocytes labeled most intensely. Metamyelocytes and polymorphonuclear (PMN) neutrophils contained about half as many grains/cell as the myelocytes. Lymphocytes, eosinophils, and basophils did not bind significant amounts of DFP under the conditions of this study while a few monocytes were lightly labeled. Granulocytes in both blood and bone marrow were labeled after intravenous injection of H^3-DFP. Relative degree of labeling was as follows: blood PMN neutrophils, 1.0; marrow PMN neutrophils, 0.3; marrow metamyelocytes, 0.2; and marrow myelocytes, 0.6. None of the other formed elements in either blood or bone marrow contained significant amounts of the label.

We are indebted to Mr. Emil Adamik for advice and assistance in preparation of the autoradiographs.

1. Athens, J. W., Mauer, A. M., Ashenbrucker, H., Cartwright, G. E., Wintrobe, M. M., *Blood,* 1959, v14, 303.

2. Mauer, A. M., Athens, J. W., Ashenbrucker, H., Cartwright, G. E., Wintrobe, M. M., *J. Clin. Invest.,* 1960, v39, 1481.

3. Mauer, A. M., Athens, J. W., Warner, H. R., Ashenbrucker, H., Cartwright, G. E., Wintrobe, M. M., in the *Kinetics of Cellular Proliferation,* Grune & Stratton, Inc., New York, 1959, 231.

4. Marsh, D. J., Neal, E., *Chem. & Ind.,* 1956, v22, 494.

5. Pelc, S. R., *Nature,* 1947, v160, 749.

6. Bond, V. P., Fliedner, T. M., Cronkite, E. P., Rubini, J. R., Brecher, G., Schork, P. K., *Acta Hematol.,* 1959, v21, 1.

7. Wachstein, M., Wolf, G., *J. Histol. & Cytochem.,* 1958, v6, 457.

8. Cohen, J. A., Warringa, M. G. P. J., *J. Clin. Invest.,* 1954, v33, 459.

9. Leeksma, C. H. W., Cohen, J. A., *ibid.,* 1956, v35, 964.

10. Tonna, E. A., Cronkite, E. P., *Stain Technol.,* 1958, v33, 255.

Received March 16, 1961. P.S.E.B.M., 1961, v107.

Haematologic and Serum Protein Alterations in the Hypothermic Rat.* (26646)

JOHN R. BEATON
Defence Research Medical Laboratories, Toronto, Ont., Canada

During studies on blood metabolite and electrolyte alterations in the hypothermic rat(1,2) we observed that, in animals with rectal temperature reduced to 15°C, there is a significant increase in hematocrit. Increased hematocrit in hypothermia has been reported by others(3,4) although the explanation is not clear. It has been suggested that the increased hematocrit may result from decreased plasma volume(5) or by splenic discharge of red cells(3). Another possible explanation would be an increase in mean corpuscular volume of erythrocytes. The present experiments were undertaken to study hematocrit, hemoglobin, blood cell and serum protein levels in hypothermic rats, seeking in particular, further information on the cause of the increased hematocrit values.

Methods. In all experiments, male albino rats of the Wistar strain, weighing 200-250 g and maintained on fox chow and water *ad libitum,* were used. Cooling was carried out as in previous experiments(1,2), by placing unanesthetized animals in individual, cylindrical screen cages under crushed ice for a period of approximately 30 minutes at which time rectal temperature was 15°C. At this rectal temperature, respiratory movements

* DRML Report No. 109-8: PCC No. D50-93-10-72.

TABLE I. Haematologic Alterations in Hypothermic (15°C Rectal Temperature) Rats. Results expressed as mean ± stand. error of the mean for 10 rats.

	Control	Hypothermic	Probability, P*
Hematocrit, %	46 ± 1.5	55 ± 1.8	<0.01
Hemoglobin, g %	13.0 ± 0.53	13.7 ± 0.35	—
Erythrocyte count, millions/mm³ blood	8.03 ± 0.12	8.48 ± 0.52	—
Mean corpuscular volume, μ^3	57.7 ± 1.3	65.4 ± 2.0	<0.01
Mean corpuscular hemoglobin content, $\mu\mu g$	16.2 ± 0.43	16.1 ± 0.52	—
Leukocyte count, thousands/mm³ blood	11.9 ± 1.0	7.6 ± 1.2	<0.01
Neutrophiles, %	24 ± 0.63	10 ± 1.7	<0.001
Lymphocytes, %	75 ± 0.63	89 ± 1.6	<0.001
Monocytes, %	0	0	—
Eosinophiles, %	<1	<1	—
Basophiles, %	0	0	—

* Calculated by application of Student's "t" test.

had almost ceased, cardiac activity, as measured by electrocardiogram, markedly reduced and the animals were lethargic. Artificial ventilation was not provided during the cooling procedure. Blood samples were withdrawn by syringe from the exposed heart (right ventricle) following intraperitoneal administration of sodium pentobarbital in 0.85% aqueous sodium chloride to provide 5 mg/100 g body weight. In the first experiment, heparin was added to blood as the anticoagulant.

In the first experiment, the following determinations were made on individual blood samples from 10 control and 10 hypothermic rats: hematocrit by the method of Wintrobe (6), hemoglobin by the method of Collier (7), erythrocyte count, leukocyte count and differential leukocyte count using Wright's stain. Mean corpuscular volume and mean corpuscular hemoglobin content were calculated from these data.

In a second experiment, blood samples from 10 control and 10 hypothermic rats were allowed to clot, the sera were separated, and total protein, albumin and globulin concentrations were determined by the method of Gornall *et al.*(8). In addition, the starch gel zone electrophoretic procedure of Smithies(9) was used to study serum proteins of 12 control and 12 hypothermic rats.

Results. The results of blood cell count, hematocrit and hemoglobin measurements are shown in Table I. The increased hematocrit of hypothermic rats(1) was confirmed and was accompanied by a significant increase in mean corpuscular volume of erythrocytes. In the hypothermic rat there was a neutropenia, a lymphocytosis and a net leukopenia. Hypothermia had no apparent effect upon erythrocyte count, hemoglobin concentration or mean corpuscular hemoglobin content.

The results of the serum protein analyses are shown in Table II. Values obtained for control animals are comparable to those obtained previously using this analytical method(10). It is apparent that hypothermia in the rat, under these conditions, results in a significant increase in total serum protein concentration and in an increased albumin: globulin ratio. Starch gel zone electrophoresis showed no qualitative differences between the sera of the control and of the hypothermic rats.

Discussion. Barbour *et al.*(5), on the basis of changes in plasma proteins and chloride, concluded there is a decrease in

TABLE II. Serum Protein Concentrations in Hypothermic Rats (15°C Rectal Temperature). Results expressed as mean ± stand. error of the mean for 10 rats.

	Control	Hypothermic	Probability, P*
Total serum proteins, g %	5.78 ± 0.044	6.30 ± 0.073	<0.001
Serum albumin, g %	3.74 ± 0.070	4.76 ± 0.044	<0.001
Serum globulin, g %	2.04 ± 0.083	1.54 ± 0.070	<0.001
Albumin: globulin ratio	1.83 ± 0.15	3.09 ± 0.28	<0.001

* Calculated by application of Student's "t" test.

plasma volume of hypothermic monkeys and rats. Our results of serum protein analyses seem to support this conclusion, as does our earlier observation(2) of a decrease in plasma water content in hypothermic rats. Swan *et al.*(3) reported increased hematocrit with little change in plasma volume which they suggested as being indicative of splenic discharge of cells. The data reported here show an increase of 13.3% in mean corpuscular volume which, although insufficient alone to account for the 19.5% increase in hematocrit, is no doubt a contributing factor. This increase in mean corpuscular volume may result, in part, from the 7.7% increase in erythrocyte water content which we have observed in hypothermic rats(2). It would appear that the increased hematocrit of our hypothermic rats is a consequence of more than one factor, including a decreased plasma volume and an increased mean corpuscular volume.

Villalabos *et al.*(11) have observed a marked decrease in leukocyte and platelet counts of hypothermic dogs and provided evidence that these decreases are due to sequestration rather than to destruction. If this is true in the hypothermic rat, the relative increase in lymphocytes and relative decrease in neutrophiles are difficult to explain unless neutrophiles are preferentially sequestered.

The increase in serum albumin concentration more than accounts for the increase in total serum proteins, since there is a decrease in serum globulin concentration. Indeed, if plasma volume is reduced in hypothermia (5), the decrease in serum globulin content is more marked than is indicated by the concentration values. This decrease might well be the cause of the increased serum albumin:globulin ratio.

Summary. As a consequence of hypothermia in the rat (15°C rectal temperature), there are significant increases in hematocrit, mean corpuscular volume, serum total protein and albumin concentrations and in the serum albumin:globulin ratio. Hypothermia does not alter erythrocyte count, hemoglobin concentration or mean corpuscular hemoglobin content. These observations are discussed in relation to the causes of the increased hematocrit. In addition, the hypothermic rat has a leukopenia with a relative decrease in neutrophiles and a relative increase in lymphocytes.

The author is indebted to Mr. T. Orme for valuable technical assistance.

1. Beaton, J. R., *Canad. J. Biochem. & Physiol.*, 1960, v38, 709.

2. ———, *ibid.*, 1961, v39, 1.

3. Swan, H., Zeavin, I., Holmer, J. H., Montgomery, V., *Ann. Surg.*, 1953, v138, 360.

4. Hegnauer, A. H., Schriber, W. J., Haterium, H. O., *Am. J. Physiol.*, 1950, v161, 455.

5. Barbour, J. G., McKay, E. A., Griffith, W. P., *ibid.*, 1943, v140, 9.

6. Wintrobe, M. M., Clinical Hematology, Lea and Febiger, Philadelphia, 1942, p44.

7. Collier, H. B., *Canad. Med. Assn. J.*, 1944, v50, 550.

8. Gornall, A. G., Bardawill, C. J., David, M. M., *J. Biol. Chem.*, 1949, v177, 751.

9. Smithies, O., *Biochem. J.*, 1959, v71, 585.

10. Beaton, J. R., Beare, J. L., White, J. M., McHenry, E. W., *J. Biol. Chem.*, 1953, v200, 715.

11. Villalabos, T. J., Adelson, E., Riley, P., in *The Physiology of Induced Hypothermia,* Nat. Acad. Sci., Nat. Research Council Publication No. 451, 1956, 186.

Received March 23, 1961. P.S.E.B.M., 1961, v107.

Effect of Chelating Agents on Uptake of Ca^{45} and Sr^{85} by Defatted Bone *in vitro*.* (26647)

JOSEPH SAMACHSON AND HILDA LEDERER (Introduced by Herta Spencer)
Division of Neoplastic Diseases, Montefiore Hospital, New York

It has been shown that the ratio of uptake of Ca^{45} to Sr^{85} by bone is lower from serum or serum ultrafiltrates than from synthetic buffered solutions(1) and it has been suggested that this lowering of the ratio is due to the presence in the serum of proteins and natural chelating agents, the latter being present in serum ultrafiltrates as well. As serum proteins bind calcium more strongly than strontium(2), it seemed possible that natural chelating agents present in serum might behave similarly. Moreover, as strong synthetic chelating agents have been proposed for removal of bone-seeking radioisotopes, it seemed of interest despite emphasis on their limitations(3) to investigate their effect on the exchange between Sr^{85} and Ca^{45} in solution and bone calcium.

The ratio of Ca^{45}/Sr^{85} uptake provides a much more reproducible index than either the Ca^{45} or Sr^{85} uptakes individually(1) and permits detection of effects which might otherwise be unnoticed. Results are presented here of a study on the effect of chelating agents and other compounds on relative bone uptake of Ca^{45} and Sr^{85} and on the use of chelating agents to remove these isotopes from bone which had taken them up by exchange. Compounds naturally present in serum, such as citrate, were studied as well as synthetic chelating agents of the polyamino polyacid type.

Materials and methods. Beef femur was used as a source of bone. The marrow was removed and the bone was crushed, ground to between 60 and 100 mesh, defatted and dried at 105°C. Approximately 150 g were thus obtained and as 100 mg portions were used in each experiment, sufficient ground bone of the same batch was available for many studies. Sr^{85} and Ca^{45} were obtained as the chloride, Sr^{85} without carrier, Ca^{45} with a specific activity of approximately 30 μc/mg.

Solutions containing Sr^{85} and Ca^{45} plus carrier were buffered with 0.04 N veronal and hydrochloric acid to give a final pH of approximately 7.3. Carriers used were $SrCl_2$ or $CaCl_2$ or a mixture of both, each carrier in a concentration of 2.5 mM/L. One hundred mg samples of ground bone were shaken gently at 37°C for 2 hours with 50 ml of solution in flasks attached to a mechanical stirrer. The effect of strong chelating agents like ethylenediaminetetraacetic acid was studied in concentrations sufficient to chelate half the alkaline earth present, *i.e.*, 1.25 mM/L with Ca or Sr carrier, 2.5 mM/L with the combined carrier. The effect of citrate was studied at concentrations from 5 to 25 mg%, of glucose at 100 mg% and of bicarbonate at 25 mM/L. The pH of the bicarbonate solutions was reduced to 7.2 or 7.3 before the experiment by passage of oxygen containing 5% CO_2, and was redetermined after shaking to insure that there had been no appreciable loss of CO_2. A combination of bicarbonate at the same concentration and phosphate at a concentration of 3 mg % was also studied.

At the end of 2 hours of shaking, the bone was ashed and analyzed for Ca, P, Sr^{85} and Ca^{45}. Sr^{85} and Ca^{45} determinations were also carried out on the original solution and on the solution after shaking with bone, while P was determined on the buffered solutions after shaking and Ca on solutions in which no Sr was present. Uptake of Sr^{85} and Ca^{45} was expressed as percent in bone compared to the amount present in the original solution. Analyses for Sr^{85} and Ca^{45} in solution were carried out to insure complete recovery of the radioisotopes and were not used to calculate uptakes, as difference between the original solution and the solution after 2 hours of shaking was much more subject to error than direct determination of the radio-

* This work was done under U. S. Atomic Energy Commission contract. A preliminary report was given at April 1959 meeting of Fed. Am. Soc. Exp. Biol.

isotope in bone. Analyses for Ca and P were likewise carried out to insure that there was complete recovery of the bone and no appreciable loss of bone into the solution.

Sr^{85} was determined in a scintillation counter of the well type, Ca^{45} by precipitation with stable Ca as previously reported (4), counting being done in a thin-window gas flow counter, stable calcium by the method of Shohl and Pedley(5), phosphorus by the method of Fiske and SubbaRow(6).

Experiments on removal of radioisotopes from bone were carried out by shaking 100 mg bone samples with Ca^{45} and Sr^{85} for 2 hours in 50 ml of buffered solutions containing no carrier and then shaking the bone samples for another 2 hours with fresh 50 ml samples of buffered solutions containing 2.5 mM/L $CaCl_2$ and a chelating agent. Only those chelating agents which had shown an effect in the previous part of the study were used in these experiments and, as before, the strong chelating agents were used in concentrations of 1.25 mM/L, citric acid in a concentration of 25 mg %. In addition to the usual analyses, the solution used for removal of the radioisotopes was also analyzed for Sr^{85} and Ca^{45}.

Studies were carried out at least in duplicate and in a few cases in triplicate or quadruplicate. To take account of the effects of variations in shaking rate and of other factors which might vary from day to day, duplicates were not usually done on the same day, except in those cases where the calcium or strontium salt of the chelate appeared to form a supersaturated solution. In such cases, duplicate experiments were performed at the same time, immediately after preparation of the chelate solution, before precipitation could take place.

In the experiments on radioisotope uptake, all compounds were studied with at least 2 of the 3 carriers (Ca, Sr, Ca + Sr). In most cases, when results with the chelating agents differed from those of the controls, the differences were obvious without statistical analysis. Nevertheless, to provide an objective test for doubtful cases, all Ca^{45}/Sr^{85} ratios obtained with each carrier were grouped together on the asumption that whether or not they were samples of different populations, they had the same standard deviation. The assumption was obviously valid for those compounds which had no effect on uptake and, as approximately half the compounds studied were of this nature, the result was to provide many replicates of a control with a good estimate of its standard deviation. The formula used was

$$\sigma^2 = \frac{1}{N-k} \sum_{j=1}^{k} \sum_{i=1}^{n_j} (x_{ij} - \bar{x}_j)^2 \qquad (7)$$

where σ is the standard deviation, x_{ij} is observation i in sample j, $\bar{x}_j$ is the mean of sample j, with n_j observations, k is the number of samples, and N total number of observations. In the present study, k is the number of compounds studied (including control), each value of j refers to a particular compound, and n_j varied from 2 to 4.

Results were considered different from the control at the 0.05 level of significance if they differed by at least 2 standard deviations. As 123 experiments were carried out, a small number of findings of significance might be expected where no significance was actually present. The possibility of such errors was reduced by considering results for a given chelating agent to be positive only when significant differences were found with all the carriers used.

Results. Table I shows the effect of strong chelating agents in reducing the ratio of Ca^{45}/Sr^{85} uptake by bone from solutions containing Ca carrier. Although the amount of uptake, as previously noted(1), was subject to considerable variation, reduction of the Ca^{45}/Sr^{85} ratio from 1.28 to 1.01 or less was a reliable indication of the effect of the chelating agents at a level of significance far beyond the 0.05 value suggested in this table. No effects were shown by glucose, lactate, gluconate, bicarbonate and the bicarbonate-phosphate mixture.

Table II shows similar effects for the same chelating agents as well as for isopropylenediaminetetraacetic acid (IPDTA) when the radioactive solution contained Ca plus Sr carrier or Sr carrier alone. The effect was even more pronounced in the presence of these carriers than in the case of Ca carrier,

TABLE I. Uptake of Ca^{45} and Sr^{85} by Bone from Solutions Containing Strong Chelates and Ca Carrier.

Chelate	Uptake by bone		
	% Ca^{45}	% Sr^{85}	Ca^{45}/Sr^{85}
None	12.8	10.0	1.28
Bis (2-aminoethyl) ether tetraacetic acid (BAETA)	12.3	12.2	1.01
Cyclohexane trans 1,2-diaminetetraacetic acid (CDTA)	11.8	13.8	.86
Ethylenediaminetetraacetic acid (EDTA)	13.4	14.1	.95
Diethylenetriaminepentaacetic acid (DTPA)	10.7	10.9	.98

Stand. dev. for Ca^{45}/Sr^{85} ratios for Ca carrier, calculated in manner given in text, was 0.04. Values of 1.19 or less, significantly different from control at 0.05 level. No effect shown by glucose, lactate, gluconate, bicarbonate, bicarbonate plus phosphate.

TABLE II. Uptake of Sr^{85} and Ca^{45} by Bone from Solutions Containing Strong Chelates and (Ca plus Sr) Carrier or Sr Carrier.

Chelate	Ca plus Sr carrier			Sr carrier		
	% Ca^{45}	% Sr^{85}	Ca^{45}/Sr^{85}	Ca^{45}	Sr^{85}	Ca^{45}/Sr^{85}
None	11.9	6.5	1.83	21.7	10.1	2.12
BAETA	8.8	7.4	1.19	13.6	9.9	1.37
CDTAδ	7.7	8.8	.88	6.5	10.1	.65
EDTA	7.7	8.1	.95	7.4	9.7	.77
DTPA	7.6	6.9	1.11	9.8	9.2	1.07
IPDTA*	8.5	10.6	.80	10.5	14.1	.75

* Isopropylenediaminetetraacetic acid.

Stand. dev. for Ca^{45}/Sr^{85} ratios, Ca plus Sr carrier, was 0.11 and all values of 1.60 or less differ significantly at 0.05 level.

Stand. dev. for Ca^{45}/Sr^{85} ratios, Sr carrier, was 0.15 and all values of 1.81 or less differ significantly at 0.05 level. No effect shown by glucose, lactate, gluconate, glutamate, aspartate, borate, lysine, glutathione, glycerophosphate, bicarbonate, bicarbonate plus phosphate.

the ratio for Sr carrier being reduced from 2.12 for the control to as little as 0.65 for cyclohexanediaminetetraacetic acid (CDTA). In Table II, the effect of the chelating agents is evident not only on the Ca^{45}/Sr^{85} ratios but on the individual radioisotope uptakes as well, that of Sr^{85} being apparently unaffected, or in the case of IPDTA possibly increased slightly, while uptake of Ca^{45} was decreased. Again no effect was shown by weak chelating agents listed below Table I or by glutamate, aspartate, borate, lysine, glycerophosphate or glutathione.

Table III shows the effect of various concentrations of citric acid and adenosine triphosphate. In the presence of Ca carrier, 10 mg % citrate reduced the ratio significantly, while in the presence of the other carriers, 25 mg% citrate was needed to obtain a definite effect. Adenosine triphosphate was effective with all 3 carriers at both concentrations used.

Table IV gives stability constants for Ca and Sr. complexes of various compounds taken from the literature(8,9,10). Those compounds which were without effect on uptake ratio had stability constants of the order of 1 or 2, while citric acid and ATP, which had a definite but moderate effect, had constants between 3 and 4 and chelating agents with the greatest effect had stability

TABLE III. Ratios of Ca^{45} and Sr^{85} Uptake by Bone from Solutions Containing Citrate or Adenosine Triphosphate with Ca (Ca plus Sr) or Sr Carriers.

Chelate	Ca^{45}/Sr^{85} ratios		
	Ca carrier	Ca + Sr	Sr carrier
None	1.28	1.83	2.12
Citrate, 5 mg %	1.20	1.88	2.18
" 10 "	1.17	1.90	2.11
" 20 "	1.03	1.71	1.92
" 25 "	1.04	1.32	1.65
ATP, to chelate ¼ of carrier	1.14	1.47	1.78
ATP, to chelate ½ of carrier	.93	1.30	1.54

Differences significant at .05 level when values for Ca carrier 1.19 or less, Ca plus Sr carrier 1.60 or less, Sr carrier 1.81 or less.

TABLE IV. Stability Constants of Compounds Used to Modify Bone Uptake.

Compounds		pK (Ca)	pK (Sr)
ATP	(9)	3.60	—
Citric acid	(8,9)	3.22	2.7
Gluconic "	(8,9)	1.21	1.00
Lactic "	(8,9)	1.07	.70
Glutamic "	(8,9)	1.43	1.37
Aspartic "	(8)	1.60	1.48
Diethyl barbituric acid	(8)	.66	.48
BAETA	(8)	10.05	9.34
CDTA	(9)	12.50	—
EDTA	(8,9)	10.59	8.63
DTPA	(8,9)	10.11	9.68
IPDTA	(10)	10.4	10.7

Stability constants vary with ionic strength, temperature and other variables but above values permit comparison of Ca and Sr binding by same compound.

constants of the order of 10. The constants cited were greater for Ca than for Sr in all cases except that of IPDTA.

Table V shows the effect of chelating agents added to solutions in order to remove Ca^{45} and Sr^{85} from bone by exchange. In the absence of chelating agents, Ca^{45} was removed from bone less readily than Sr^{85}. Addition of chelating agents to the wash solutions increased the relative removal of Ca^{45} in all cases listed in the table except that of citric acid, which showed a doubtful effect. There appeared to be either unchanged or slightly increased removal of Ca^{45}, as shown in the first column of the table, accompanied by decreased removal of Sr^{85}.

Discussion. From the results obtained, it appears that the Ca^{45}/Sr^{85} uptake ratio found under the present experimental conditions may be used as a criterion of the difference in binding of Ca and Sr by a chelating agent. All of the strong chelating agents used in this study, except IPDTA, are reported to have greater stability constants for Ca than for Sr, and in view of the fact that IPDTA decreased relative Ca^{45} uptake at least as well as EDTA, CDTA and the other strong chelates, the stability data reported for this compound may well be erroneous. The uptake data for IPDTA are difficult to explain on the basis of a greater stability constant for Sr.

At the concentrations used, practically all the chelating agent was in combined form. In the presence of Ca or Ca plus Sr carrier, the calcium complex was chiefly formed. With EDTA, for example, whose Ca and Sr stability constants differ by 2 units, the amount of Ca chelate formed was approximately 100 times that of the Sr chelate. With other chelating agents, ratio of Ca chelate to Sr chelate was lower but still much greater than 1.

The preferential sequestration of Ca ions resulting from the greater stability constant for Ca resulted in a decrease in the uptake ratio of Ca^{45}/Sr^{85} but not in any obvious relation to the decrease of Ca ions. In the case of Ca carrier, where approximately half the calcium was bound and little of the Sr^{85}, the greatest decrease in ratio was shown by CDTA, which gave a value approximately two-thirds that of the control. In the case of Sr carrier, where all the Ca was bound and half the Sr^{85}, the ratio was reduced to about one-third that of the control.

The reason for a lack of parallelism in Ca^{45} exchange and Ca ion concentration must be sought in the nature of the exchange reaction. Neuman and Neuman(11) believe that with bone mineral, the first step is an exchange of ions between bulk solution and hydration shell of the bone crystal. Chelation obviously affects this exchange, but as chelated Ca and Sr may also penetrate the hydration shell, the net effect on this first step may be minor. The second step suggested(11) is an exchange between hydration shell and crystal surface itself and here the effect of chelate may be more pronounced. Ca ions in the

TABLE V. Ca^{45} and Sr^{85} Removed from Bone by Solutions Containing Stable Ca and Chelates.

Chelate	% Ca^{45} removed	% Sr^{85} removed	Ca^{45}/Sr^{85}
None	45.5	49.7	.92
EDTA	43.7	28.7	1.52
DTPA	52.4	40.9	1.28
BAETA	53.4	44.7	1.20
IPDTA	55.2	31.7	1.71
Citrate	36.8	38.6	.96
ATP	38.7	32.7	1.19

Ca^{45} values significantly different at .05 level outside of range 38.7 to 52.3.

Sr^{85} values significantly different at .05 level outside of range 45.7 to 53.7.

Ca^{45}/Sr^{85} ratios significantly different at .05 level above .96.

crystal surface may exchange directly with Ca ions in the hydration shell and therefore more rapidly than with chelated Ca.

Subsequent steps, which involve interchange between surface positions and crystal interior, along with possible remodeling and recrystallization, are of no importance in the short periods here under consideration. However, even the first 2 steps overlap and complications are introduced, as Dallemagne and his coworkers have emphasized(12,13,14), by the organic phase of bone. Although Ca^{45} and Sr^{85} do not concentrate in the organic phase, it is the medium in which ions diffuse (12). Ca and Sr ions are bound to some extent by this organic phase(1), so that diffusion through it is not merely a physical process but is accompanied by continual binding and release of alkaline earth ions. Chelating agents affect this process by lowering the concentration of free alkaline earth ions but may to some extent supplement it with the diffusion of Ca and Sr chelates.

In the overall picture, therefore, a net effect is evident even with such moderately strong chelating agents as citric acid and ATP. Apparently, however, compounds which form complexes with stability constants for Ca and Sr too close together do not affect the ratio. (It may be noted that the stability constants for diethylbarbituric acid are below 1 and that use of this compound as a buffer, therefore, does not affect the results.) As citric acid and ATP are both present in serum, although in smaller amounts than here used, and as other natural chelating agents may also be present, results support the assumption that natural chelating agents are at least partly responsible for the low Ca^{45}/Sr^{85} uptake ratio from serum and ultrafiltrate compared to uptake from synthetic buffered solutions.

It has previously been shown that the presence of phosphate does not affect the Ca^{45}/Sr^{85} ratio(1). The lack of effect of bicarbonate, as well as of bicarbonate-phosphate mixtures, is of interest, as calcium phosphate and calcium phosphate-bicarbonate complexes may exist in serum(15). If they do exist, their failure to affect the ratio may be due to the low order of the calcium stability constant, the closeness of values for Ca and Sr stability, or both factors.

The effect of strong chelating agents on the relative removal of Sr^{85} and Ca^{45} by exchange is important. It has been shown that because of the preferential chelation of Ca compared to Sr, the sodium salt of EDTA actually retards renal elimination of Sr^{85} (16). Although this unfavorable effect can to some extent be overcome by using the Ca salt instead of the Na salt, and substituting other chelating agents for EDTA, the preferential chelation of Ca still causes difficulties. It is now evident that strong chelates also decrease relative removal of Sr^{85} from bone by exchange. It must be emphasized that these data obtained *in vitro,* are not directly applicable to the living organism, where accretion of bone containing radioisotopes takes place, and dissolution of bone crystals is needed to remove the radioisotopes. However, as chelates appear to be potentially most effective shortly after administration of a dose, before much accretion of bone has taken place, the attempted removal of radiostrontium with chelating agents must still contend with the unfavorable effect of chelates on exchange, unless chelating agents with stability constants for Sr approaching or exceeding those for Ca are developed.

Summary. The presence of chelating agents in buffered solutions affected the relative uptake of Ca^{45} and Sr^{85} by defatted bone powder. Strong chelating agents, like ethylenediaminetetraacetic acid and cyclohexanediaminetetraacetic acid, decreased the ratio of Ca^{45}/Sr^{85} uptake considerably in presence of Ca, Ca plus Sr, or Sr carrier. Citrate and adenosinetriphosphate had similar but weaker effects. No effect was shown by glucose, lactate, gluconate, bicarbonate, bicarbonate plus phosphate, glutamate, aspartate, borate, glycerophosphate, lysine or glutathione. Those compounds which showed no effect had stability constants for Ca of less than 3. Strong chelating agents also decreased the relative amount of Sr^{85} removed from defatted bone powder by exchange. Results indicate that natural chelating agents may be partly responsible for the low Ca^{45}/Sr^{85} uptake ratio by bone from serum com-

pared with uptake from synthetic inorganic solutions and emphasize the difficulty of removing Sr^{85} from bone with chelating agents now available.

1. Samachson, J., Lederer, H., *Arch. Biochem. Biophys.,* 1960, v88, 355.
2. ———, PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 867.
3. Schubert, J., *Atompraxis,* 1958, v11, 393.
4. Blau, M., Spencer, H., Swernov, J., Laszlo, D., *Science,* 1954, v120, 1029.
5. Shohl, T. A., Pedley, F. G., *J. Biol. Chem.,* 1922, v50, 537.
6. Fiske, C. H., SubbaRow, T. J., *ibid.,* 1925, v66, 375.
7. Wilson, E. B., Jr., *An Introduction to Scientific Research,* McGraw-Hill, New York, 1952, p245.
8. Bjerrum, J., Schwarzenbach, G., Sillen, L. G., *Stability Constants,* Part I, The Chemical Soc., London, 1957.
9. Chaberek, S., Martell, A. E., *Organic Sequestering Agents,* Wiley, New York, 1959.
10. Smith, R. L., *The Sequestration of Metals, Macmillan, New York,* 1959, p82.
11. Neuman, W. F., Neuman, M. W., *Chemical Dynamics of Bone Mineral,* U. of Chicago Press, Chicago, 1958.
12. Dallemagne, M. J., Baud, C. A., Morgenthaler, P. W., *Histochemie,* 1959, v1, 185.
13. Dallemagne, M. J., *Arch. Intern. de Phys. et. de Biochim.,* 1958, v66, 84.
14. ———, *Acta Physiol. Pharm. Neerlandica,* 1957, v6, 469.
15. Greenwald, J., *J. Biol. Chem.,* 1945, v161, 697.
16. Spencer, H., Samachson, J., Laszlo, D., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 565.

Received March 24, 1961. P.S.E.B.M., 1961, v107.

Four Newly Recognized Adenoviruses. (26648)

LEON ROSEN, SAMUEL BARON, AND JOSEPH A. BELL (Introduced by R. J. Huebner)
Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, and Laboratory of Viral Immunology, Division of Biologics Standards, NIH, Bethesda, Md.

During a longitudinal study of the microbial experiences of children in an institution in Washington, D. C.(1) more than 50 strains of adenoviruses were isolated which could not be identified as any of the known types(2,3). Further study revealed that these viruses could be grouped into the 4 previously unrecognized serotypes which are described in this report.

Materials and methods. Prototype adenovirus strains were obtained from Drs. W. P. Rowe and S. D. Bell, Jr. The "S-1058" strain of type 7A was used as the representative of type 7. Neutralization tests were carried out in Rhesus kidney cell cultures(2) employing "procedure 2" of Rowe, *et al.*(4). Hemagglutination and hemagglutination-inhibition (HI) tests were performed as described previously(5). Rabbit antisera were used in both neutralization and HI tests(5). Complement-fixation tests were done by a standard technic which has been used with a number of viral antigens(4).

Throat and anal swabs were collected, stored, and inoculated by the methods outlined previously(6). Isolates were recovered in a variety of tissue cultures of human origin maintained for varying periods of time on several different kinds of media. At least one strain of each type, however, was isolated in either HeLa or KB cells. The latter tissues had been grown on a medium consisting of Eagle's basal medium with 10% inactivated human serum and were maintained on medium No. 199 with 5% inactivated chicken serum. After inoculation, KB and HeLa cultures were observed for cytopathic effects (CPE) for 7 to 12 days, when at least one blind passage was made. Maintenance fluid was usually changed every 2 or 3 days, but in some instances changes were omitted in cultures observed for only 7 days.

Results. The viruses described here appeared to be less readily isolated from a given specimen in this study than did adenovirus types 1, 2, 3, and 5. In general, the CPE of the new serotypes was slower to appear than that of the latter viruses and in

many cases a definite CPE was not seen until one or 2 blind passages had been made. The nature of the CPE of the new serotypes in unstained cultures of both Rhesus kidney and KB cells was also distinctly different from that of types 1, 2, 3, and 5, but was indistinguishable from that of types 9, 10, and 13. As was the usual experience with types 9, 10, and 13 in this study, and again in contrast to the findings with types 1, 2, 3, and 5, isolates of the new viruses were obtained only from anal specimens. Throat and anal specimens taken from the same child at the same time were almost always tested in parallel.

Some of the children yielding isolates of the new serotypes had minor illnesses of a variety of types. However, minor illnesses were extremely prevalent in this particular study population and the data available were not sufficient to determine if the newly recognized adenoviruses were etiologically associated with any of them.

After all untyped adenovirus isolates had been grouped into 4 serotypes by means of HI tests, one strain of each type was selected as the prototype and all further studies were carried out with these strains unless otherwise noted. The origin of the 4 prototype strains was as follows.

The strain selected as the prototype of the virus which will be referred to as BP-1 was isolated from an anal specimen collected from a 16-months-old female Negro child on Aug. 1, 1956. Four other strains of this type were isolated from the same study population, one in 1955 and the others in 1956.

The strain selected as the prototype of the virus which will be referred to as BP-2 was isolated from an anal specimen collected from a 9-months-old male Negro child on July 16, 1956. Many other strains were isolated from the same population in 1956, 1957, 1958, and 1959 as well as from children in Saudi Arabia(7). This serotype has been referred to previously as BAR-2(8).

The strain selected as the prototype of the virus which will be referred to as BP-4 was isolated from an anal specimen collected from a 9-months-old female Negro child on Feb. 24, 1958. Five other strains of this type were isolated from the same population, one in 1957 and the others in 1958.

TABLE I. Homologous Hemagglutination-Inhibition Titers of Prototype Antisera.

Virus type	Homologous titer	Virus type	Homologous titer
1	320*	15	640
2	320	16	320
3	5120	17	640
4	320	18	†
5	320	19	80
6	320	20	80
7	2560	21	160
8	1280	22	1280
9	2560	23	2560
10	640	24	160
11	80	BP-1	80
12	†	BP-2	5120
13	1280	BP-4	320
14	320	BP-5	80

* Reciprocal of serum dilution.
† Homologous HI titer not obtainable because virus does not hemagglutinate. Neutralization titers of types 12 and 18 sera were $\geq 1:800$ and 1:80 respectively.

The strain selected as the prototype of the virus which will be referred to as BP-5 was isolated from an anal specimen collected from a 30-months-old male Negro child on April 23, 1958. Four other strains of this type were isolated from the same population, all in 1958.

With the exception of BP-1 virus, a rise in neutralizing antibody was demonstrated with each of the new types in one or more of the children yielding isolates. Only a single pair of sera from a child with a BP-1 virus isolate was available and no neutralizing antibody was detected in either serum. Two strains of BP-1 virus were reisolated, however, from the original specimens indicating that the viruses had originated there and not in the tissue culture system.

A sample of each of the new serotypes was found to have the same titer in Rhesus kidney cell cultures after treatment with 20% ethyl ether for 18 hours at 4°C as did a control sample similarly treated with 0.85% NaCl.

Using each of the new serotypes as an antigen, a significant rise in complement-fixing antibody titer was demonstrated in paired sera from three individuals each of whom was known to have been infected with a different previously described adenovirus.

None of the new serotypes could be passed serially in Rhesus kidney cultures and their CPE as seen in unstained Rhesus kidney cultures in primary passage was distinctly different from that of the chimpanzee and monkey adenoviruses described by Rowe *et al.* (2).

Each of the new prototype viruses was set up in neutralization tests against antisera of each of the other human serotypes. The homologous titers of these sera ranged from 1:80 to 1:320 or greater and they were used initially at a dilution of 1:5 or 1:20. Conversely, antisera for each of the new types was tested against each of the other viruses. The titer of the BP-1 serum was 1:80 and that of the BP-2, BP-4, and BP-5 sera was 1:320 or greater. The BP-1 serum was used initially at a dilution of 1:5 and the others at a dilution of 1:20. The heterologous neutralizations observed were as follows. BP-2 antiserum had a titer of greater than 1:20 but less than 1:80 against both type 9 and BP-4 virus. BP-1 antiserum had a titer of greater than 1:20 but less than 1:80 against type 10 virus and a titer of 1:80 against type 15 virus. The antiserum for type 15 virus, which had a homologous titer of 1:320, had a titer of greater than 1:20 but less than 1:80 against BP-1 virus.

Each of the new viruses was also set up in HI tests against antisera of each of the other human adenovirus serotypes. Conversely, antisera of each of the new types were tested against each of the other viruses with the exception of types 12 and 18 (which do not hemagglutinate). Types 20, 21, BP-1, and BP-5 agglutinate Rhesus but not rat erythrocytes, and types 19, 22, 23, 24, BP-2, and BP-4 agglutinate rat cells completely. The homologous HI titers of the sera used are shown in Table I. No heterologous inhibition was observed between the 4 new types, or between these types and the 24 previously described serotypes, when the sera were used at a dilution of 1:10.

Discussion. The data presented indicate that the 4 virus serotypes described belong in the adenovirus family and that they were derived from human beings. With the exception of the BP-1 virus, each appears to be clearly distinct from the 24 previously described human adenoviruses by both neutralization and HI tests.

Although the BP-1 serum had the same neutralizing titer against type 15 virus as it did against its homologous virus, it is proposed that BP-1 virus be considered a distinct serotype, rather than a "prime" strain of type 15, for the following reasons. Firstly, BP-1 virus does not agglutinate rat erythrocytes whereas type 15 virus agglutinates these cells to high titer. Secondly, no crossing was observed between BP-1 and type 15 in reciprocal HI tests. Types 7 and 7A(2) are indistinguishable in reciprocal HI tests (5). Finally, certain problems have been encountered with adenovirus neutralization tests which suggest that data obtained by this technic should be considered with some caution. For example, adenovirus isolates have been encountered which were *doubly* neutralized by antisera of types 3 and 7, 14 and 16, and 9 and 15 respectively. In each instance the isolates had the hemagglutinating characteristics of only one virus of the pair and were inhibited only by the serum of that same virus(5). In the publication (2) which describes adenovirus type 15 it was noted that the antiserum for this virus had a titer against adenovirus type 4 only 4-fold lower than its homologous titer. Type 4 virus is clearly different from type 15 in CPE, hemagglutinating properties, and other characteristics.

After reviewing the data presented here, the Adenovirus Committee of the Nat. Inst. of Allergy and Infectious Diseases recommended that BP-1, BP-2, BP-4, and BP-5 viruses be designated adenovirus types 25, 26, 27, and 28 respectively.

Summary. A number of strains of adenoviruses recovered from anal swabs of children in Washington, D. C., were found to belong to the 4 previously unrecognized adenovirus serotypes (BP-1, BP-2, BP-4, BP-5) described in this report. The Adenovirus Committee of the Nat. Inst. of Allergy and Infectious Diseases has recommended that these viruses be designated adenovirus types 25, 26, 27, and 28 respectively.

The authors are indebted to Mrs. J. Hovis, Mr.

R. Low, Mr. H. Turner, and Mrs. M. Wormald for valuable technical assistance.

1. Bell, J. A., Huebner, R. J., Rosen, L., Rowe, W. P., Cole, R. M., Mastrota, F. M., Floyd, T. M., Chanock, R. M. In preparation.

2. Rowe, W. P., Hartley, J. W., Huebner, R. J., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 465.

3. Bell, S. D., Jr., Rota, T. R., McComb, D. E., *Am. J. Trop. Med. & Hyg.*, 1960, v9, 523.

4. Rowe, W. P., Huebner, R. J., Hartley, J. W., Ward, T. G., Parrott, R. H., *Am. J. Hyg.*, 1955, v61, 197.

5. Rosen, L., *ibid.*, 1960, v71, 120.

6. Rosen, L., Johnson, J. H., Huebner, R. J., Bell, J. A., *ibid.*, 1958, v67, 300.

7. Bell, S. D., Jr., McComb, D. E., Murray, E. S., Chang, R. S., Snyder, J. C., *Am. J. Trop. Med. & Hyg.*, 1959, v8, 492.

8. Rosen, L., *Virology*, 1958, v5, 574.

Received April 10, 1961. P.S.E.B.M., 1961, v107.

Effect of a Necrogenic Yeast Diet on Viral Hepatitis (M.H.V.$_3$) in Mice.* (26649)

BORIS H. RUEBNER AND KATSUMI MIYAI† (Introduced by W. H. Sheldon)
Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Md.

Certain yeast diets have long been known to produce liver necrosis in rats(1). In mice DeWitt and Schwarz(2) have recently produced necrosis affecting the liver, heart and some other organs by using a diet in which dried Torula yeast was the sole source of protein. They also showed that these lesions can be prevented by supplementation with inorganic selenium or alpha tocopherol. In chicks a Torula yeast diet produces exudates in muscle, fat and skin which can be prevented by the same supplements(3). We have investigated the effect of the necrogenic diet on experimental hepatitis produced in mice by M.H.V.$_3$ virus(4). It had previously been shown that an increase in protein intake greatly reduced the mortality from this infection. This effect could not be reproduced by individual amino acids(5) or selenium.‡

Methods. Female weanling Swiss Webster mice were used. Repeated blood examinations had shown these mice to be free of Eperythrozoon coccoides, a protozoan which enhances the severity of the hepatitis. The principal constituents of the experimental diets are shown in Table I. The necrogenic diet (Diet I) was similar to that of DeWitt and Schwarz(2). Diets II and III were identical with Diet I except for supplementation with selenium and alpha tocopherol respectively. Diet IV was similar to Diet I except for substitution of casein for yeast. Since only about half the yeast consists of protein, the 15% of casein in Diet IV supplied approximately the same amount of protein as the 30% of yeast in Diet I.

Groups of mice aged 17 days were fed unrestricted quantities of the various diets. Unlimited fresh water was supplied. The animals were weighed twice weekly. After approximately 4 weeks a few deaths started to occur in Group I, the members of which were fed the necrogenic yeast diet. After 31 days half the animals of Group I and most of those on Diets II, III and IV were inoculated intraperitoneally with 0.1 ml of the M.H.V.$_3$ virus preparation(7). This consisted of a 10% suspension in Gey's balanced salt solution of mouse liver recently obtained from infected animals and stored at −30°C. Cages were inspected daily and dead mice removed. Some in each group were autopsied.

Results. In the first experiment mice fed the necrogenic diet (Diet I) were compared with animals receiving a diet identical apart from supplementation with alpha tocopherol (Diet II). Both groups were compared with

* This investigation was aided by U. S. Public Health Service Research Grant from Nat. Inst. Health, Bethesda.

† Fellow supported by U. S. Public Health Service Training Grant.

‡ Unpublished.

TABLE I. Composition of Experimental Diets (% by Weight).

Diet	Torula yeast, %	Casein, %	Dextrin, %	Stripped lard, %	α-tocopherol, %	Sodium acid selenite, mg %	Salts and vitamins, %
I	30	—	60	5	—	—	to 100%
II	30	—	60	5	.05	—	" "
III	30	—	60	5	—	.04	" "
IV	—	15	75	5	.05	—	" "

Torula yeast was kindly supplied by Lake States Yeast and Chemical Division, St. Regis Paper Co., Rhinelander, Wis.; "vitamin-free" casein and white dextrin were obtained from Nutritional Biochemicals Corp.; stripped lard from Distillation Products Industries, Rochester, N. Y. Salts and vitamins included cod liver oil, 1%; salt mixture No. 2 USP, 2.5%; vitamin mixture(6), 1%; choline chloride, 0.5%.

TABLE II. Number of Mice Dying Each Day after Injection of M.H.V.$_3$ Virus.

	Day								Total deaths	Mortality, %
	1	2	3	4	5	6	7	8		
30 mice on Diet I	1	0	14	6	4	0	0	2	27	90
28 " " " II	0	0	4	4	3	0	2	0	13	46
32 " " " IV	0	0	3	5	3	2	0	1	14	44

mice fed a casein diet approximately identical in protein concentration (Diet IV). All the 3 diets were adequate for growth. The mean weight increase produced by Diet I, the necrogenic yeast diet, was 6.1 g, slightly less than the 7.1 g gained by animals in Group II the diet of which was supplemented with alpha tocopherol. Animals receiving the casein diet (Diet IV) gained 13.4 g, approximately twice as much as those fed the yeast diets (Diets I and II).

The number of mice dying each day after infection is shown in Table II. The mortality of mice fed necrogenic diet (Diet I) was approximately double that of animals given selenium supplemented yeast diet (Diet II). This difference was statistically highly significant ($X^2 = 12.4$; $p<0.001$). Substitution of casein for yeast (Diet IV) reduced mortality from experimental hepatitis to a similar extent. Among 27 uninjected control animals receiving necrogenic diet (Diet I) there were 2 deaths during the same period. No deaths occurred among the uninjected control animals on Diet II and IV.

In the second experiment Diet I was compared with Diet II and III. Mean weight increase produced by the necrogenic diet (7.0 g) was again rather less than the gain by animals on the diets supplemented with alpha tocopherol (9.6 g) and selenium (8.5 g).

Number of mice dying each day after infection is shown in Table III. Mortality of animals given Diet I was again significantly higher than that of animals fed Diet II. Selenium proved equally effective in reducing the mortality from M.H.V.$_3$ infection (Diet III). During the same period there was one death among 30 uninjected control mice receiving Diet I. No deaths occurred among the uninjected control animals fed Diets II and III.

Discussion. The principal finding in this study was that a Torula yeast diet(2) which produced dietary necrotic degeneration in mice also increased susceptibility of these animals to experimental viral hepatitis. Alpha tocopherol and selenium which are

TABLE III. Number of Mice Dying Each Day after Injection of M.H.V.$_3$ Virus.

	Day								Total deaths	Mortality, %
	1	2	3	4	5	6	7	8		
30 mice on Diet I	0	0	5	15	7	1	0	1	29	96
45 " " " II	0	0	2	12	13	5	1	0	33	73
45 " " " III	0	0	1	9	7	11	5	0	33	73

capable of preventing the dietary necrosis (2) were effective also in reducing mortality of mice fed the necrogenic diet and infected with M.H.V.$_3$ virus. In chicks a Torula yeast diet produces multiple exudates. Like necrotic degeneration in mice these lesions can be prevented by both alpha tocopherol and selenium(3). Our experiments have provided more evidence that very small amounts of selenium can often replace Vit. E as an essential nutrient. We have also shown that mortality of mice infected with experimental viral hepatitis and receiving a casein diet supplying 15% of protein is similar to that of animals receiving a yeast diet of similar concentration and supplemented with alpha tocopherol. Although casein and yeast had a similar effect on mortality, casein was considerably more effective in stimulating growth than yeast supplemented with alpha tocopherol. This result confirms previous observations suggesting that the growth-promoting and anti-infective effects of protein are independent of one another(5).

Summary. A necrogenic Torula yeast diet increased the susceptibility of mice to experimental viral hepatitis. Supplementation of this diet with alpha tocopherol or selenium reduced the mortality of this infection. The susceptibility of mice fed a casein diet resembled that of animals receiving a yeast diet of similar protein content and supplemented with alpha tocopherol.

1. Himsworth, H. P., *The Liver and its Diseases,* 2nd Edit., Cambridge, Mass., 1950, p120.

2. DeWitt, W. B., Schwarz, K., *Experientia,* 1958, v14, 28.

3. Dam, H., Nielson, G. K., Prange, I., Søndergaard, E., *ibid.,* 1957, v13, 493.

4. Ruebner, B., Bramhall, J. L., *Arch. Path.,* 1960, v69, 190.

5. Ruebner, B., Miyai, K., *J. Lab. and Clin. Med.,* 1961, in press.

6. Meader, R. D., Williams, W., *Am. J. Anat.,* 1957, v100, 167.

7. Dick, G. W. A., Niven, J. S. F., Gledhill, A. W., *Brit. J. Exp. Path.,* 1956, v37, 90.

Received April 4, 1961. P.S.E.B.M., 1961, v107.

Precursors of Etiocholanolone and Androsterone in Adrenal Carcinoma. (26650)

MORTIMER B. LIPSETT (Introduced by Roy Hertz)
Endocrinology Branch, National Cancer Institute, Bethesda, Md.

Most patients with adrenal carcinoma excrete increased amounts of etiocholanolone (E) and variably increased amounts of androsterone (A) resulting in an E/A ratio well above the normal range(1,2). This has been interpreted to mean that some of the E originates from C_{21}-steroids whose metabolism leads to 17-ketosteroids (17-KS) of the 5β-series predominantly. Since the relative amounts of 5α- and 5β-17-KS may be related not only to the precursors but to the metabolic transformations of any particular precursor, tritium-labeled dehydroepiandrosterone (DHA) has been used to study the origin of a urinary A and E in patients with adrenal carcinoma. The secretion rate of DHA was also estimated in these patients.

Materials and methods. Five mg of DHA acetate were added to one millicurie of 7-tritium-DHA acetate (New England Nuclear Corp.), the material chromatogrammed in phenyl Cellosolve/heptane (I), eluted, and hydrolyzed with 0.067 N NaOH. It was then successively chromatogrammed in ligroin/propylene glycol (II), and isooctane/methanol:water, 10/8:2 (III). The DHA then had a specific activity (S.A.) of 51 $\times$ 10^6 cpm/mg.

The samples were counted in the Packard Tri-Carb liquid scintillation spectrometer with an efficiency of approximately 15%. Sufficient counts were accumulated to give a standard error of no more than 5%.

Urine was collected for 48 hours after intravenous injection of 2.1 $\times$ 10^6 cpm of DHA. The urines were treated as previously

TABLE I. Case Material.

Patient	Age	Sex	Diagnosis	Clinical syndrome	Site of metastases	Urinary excretion, mg/24 hr 17-KS	17-OHCS	THS
Wh	27	♀	Chorioca	Good health	—	9	6.2	.2
Cha	23	♀	"	"	—	11	5.5	.2
Sc	7	♂	Adrenal ca	Virilism	Lung	44	4.3	.2
Che	49	♀	"	Cushing's & virilism	Lung, liver	480	170	56
Ma	37	♀	"	"	Lung, abdomen	58	15	4.2
St	55	♀	"	"	Lung	46	14	3.5

described(3) and DHA separated from A and E by digitonin precipitation. The ketosteroids were then chromatogrammed in systems II and III, acetylated, rechromatogrammed in I, and measured by a micro-Zimmerman technic(4). The methods used in this laboratory for 17-KS, 17-hydroxycorticoids (17-OHCS), and tetrahydro-compound S (THS) have been listed(3).

The secretory rate of DHA was calculated by the formula:

$$\text{Secretory rate} = \frac{\text{cpm DHA injected}}{\text{S.A. of urinary DHA}}.$$

This is a maximal value since less than 100% of the isotope is excreted in 48 hours(5). Since the S.A. of A and E should equal the S.A. of DHA if they were completely derived from DHA, a decrease in S.A. could result only through the contribution of unlabelled precursor to urinary A and E. The percentage of A derived from DHA must then be $\frac{\text{S.A.}_{\text{A}}}{\text{S.A.}_{\text{DHA}}} \times 100$, and similarly for E. The errors in the methods are such that the S.A. of the urinary ketosteroids had a standard error of ± 8%.

Six patients were studied, 2 ovariectomized women and 4 patients with metastatic adrenal carcinoma. The 2 ovariectomized women had been treated for choriocarcinoma previously, but were in good health and apparently free of disease at the time of study. Pertinent features of these cases are presented in Table I.

Results. (Table II). In both normal subjects, S.A._{A} and S.A._{E} did not differ significantly from S.A._{DHA}. Thus the fraction of A and E derived from precursors other than DHA was negligible. Estimated secretion rates of DHA were 7 mg and 11 mg daily.

The patients with adrenal cancer excreted large amounts of E and A. In 3 of them, the E/A ratio was above normal (2.6, 4.1, 5.2). The S.A._{A} did not differ significantly from the S.A._{DHA} in 3 of the 4 patients. Only in the patient with the highest excretion of 17-KS (Che) could it be shown that as much as 19% of the androsterone was not derived from DHA. In contrast, in 3 of the 4 patients with adrenal cancer, the S.A._{E} was lower than S.A._{DHA} so that only 35%, 34%, and 76% of the E was derived from DHA.

The calculated daily secretion rates of DHA varied from 28 mg to 390 mg in the patients with carcinoma. There was a rough correspondence between these secretion rates and urinary excretion of DHA.

TABLE II. Steroid Excretion, Specific Activities and Derived Data.

Patient	Urinary excretion, mg/24 hr* A	E	DHA	E/A	Specific activity, cpm/μg A	E	DHA	DHA secretion rate, mg/24 hr	% derived from DHA A	E
Wh	1.7	2.3	.6	1.5	93	95	98	11	95	97
Cha	1.9	1.8	.9	1.0	161	139	150	7	107	93
Sc	11	14	18	1.3	35	34	38	28	92	90
Che	27	112	161	4.1	2.2	.92	2.7	39	81	34
Ma	4.2	22	21	5.2	20	9.1	26	41	77	35
St	3.5	9.1	15	2.6	34	25	33	32	103	76

* A = Androsterone. E = Etiocholanolone. DHA = Dehydroepiandrosterone.

Discussion. The method for measuring the extent to which a steroid metabolite is derived from a precursor was described by Vande Wiele and Lieberman(5) who showed that, in the normal adult, DHA is the precursor of the urinary A and E. The results in the 2 ovariectomized women are in accord with this. In 3 of the 4 cases of adrenal carcinoma, however, urinary E must have originated from both DHA and other precursors, these other precursors accounting for as much as ⅔ of the E in 2 of the patients. It is significant that the 3 patients with $S.A._{E} < S.A._{DHA}$ were those with excess corticoid production as shown by the excretion of 17-OHCS and THS.

The 17-KS produced by the metabolism of 17α-hydroxyprogesterone(6,7) and compound S(8) are predominantly of the 5β-series, and it has been suggested(2) that compound S may be an important precursor of urinary E in adrenal cancer. In a series of 10 patients with adrenal carcinoma, both THS and pregnanetriol were generally found in large amounts when the cancer was producing corticoids (Lipsett and Wilson, unpublished data). This implies that 17α-hydroxyprogesterone and compound S were not being utilized normally in biosynthesis of cortisol. Thus it seems reasonable to suppose that the large amounts of E in adrenal cancer are due not only to over-production of DHA but to the metabolism of 17α-hydroxyprogesterone and compound S as well, in many of the patients.

This viewpoint is supported by the study in Sc. This boy had no evidence of Cushing's syndrome and excretion of THS and 17-OHCS was normal. The E/A ratio was 1.3 although both steroids were excreted in large amounts. The S.A. of A and E did not differ significantly from $S.A._{DHA}$, and thus only small quantities of A and E could have been derived from steroids other than DHA.

With respect to A, the specific activity was significantly lower than that of D in 2 of the 4 cases. The unlabelled A may have arisen from 17α-hydroxyprogesterone and compound S as discussed, and a further contribution of androstenedione to urinary A and E seems possible(9). Degree of dilution of the labelled A was not of the same magnitude as that of E. This would not be expected in view of the above considerations.

The secretion rate of DHA in the 2 normal subjects and in 4 others(5) suggests that DHA is quantitatively, at least, an important secretory product of the adrenal cortex. The accuracy of the secretion rates in adrenal cancer may be less than in the normal, since it seems possible that equilibration of the injected isotope with the large pool may not be sufficiently rapid with respect to its excretion. If such an effect occurred, however, the true secretion rates would be even higher than those estimated.

Summary. The precursor of urinary androsterone and etiocholanolone was shown to be dehydroepiandrosterone in 2 ovariectomized women. In 3 women with Cushing's syndrome due to adrenal carcinoma, there were other major precursors of etiocholanolone and, to a lesser extent, of androsterone. It was suggested that these other precursors were 17α-hydroxyprogesterone and compound S. In one patient with adrenal cancer who excreted only 11-deoxy-17-ketosteroids, etiocholanolone was derived almost entirely from dehydroepiandrosterone. The secretion rate of DHA was 7 and 11 mg daily in 2 ovariectomized women. In 4 patients with adrenal carcinoma, it ranged from 28 mg to 390 mg daily.

I wish to thank Drs. Seymour Lieberman and Bernt Hökfelt for stimulating discussion and helpful criticism. Mrs. Barbara Riter contributed excellent technical assistance.

1. Gallagher, T. F., *Cancer Res.,* 1957, v17, 520.

2. Vande Wiele, R., Christy, N. P., Lieberman, S., Jailer, J. W., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 520.

3. Lipsett, M. B., Damast, B., *ibid.,* 1958, v99, 285.

4. Wilson, H., *Arch. Biochem. Biophys.,* 1954, v52, 217.

5. Vande Wiele, R., Lieberman, S., in *Biological Activities of Steroids in Relation to Cancer,* G. Pincus and E. P. Vollmer, Eds., 1960, Academic Press, N. Y. and London, p93.

6. Jailer, J. W., Gold J. J., Vande Wiele, R., Lieberman, S., *J. Clin. Invest.,* 1955, v34, 1639.

7. Axelrod, L. R., Goldzieher, J. W., *J. Clin. En-*

docrinol. Metab., 1960, v20, 238.
8. Birke, G., *Acta Endocrinol.*, 1954, v15, 17.
9. Hirschmann, H., De Courcy, C., Levy, R. P., Miller, K. L., *J. Biol. Chem.*, 1960, v235, P.C. 48.

Received April 27, 1961. P.S.E.B.M., 1961, v107.

Effect of Certain Liquid Organopolysiloxanes on Cholesterol Atherosclerosis of the Rabbit.* (26651)

FRANK GOLLAN (Introduced by Wilson C. Grant)
Veterans Administration Hospital and University of Miami School of Medicine, Coral Gables, Fla.

It has been postulated that the stability of lipids in emulsion form depends on maintenance of a sufficient surface charge, so that deposition of lipid from plasma at certain sites on the linings of the blood vessels might well result from a lack of surface active agents in plasma(1). Fatty acids are anionic surface active agents and their surface activity is increased by presence of double bonds in the fatty acid radicle(2). Thus, the least saturated fatty acid is the most effective as a charged surface active agent. The pronounced lipemia and cholesterolemia producing effect of a surface active detergent like Triton WR-1339 has been thoroughly explored(3), whereas hydrophobic surface active agents like the silicone fluids have received little attention. Dimethylpolysiloxanes, like DC 200 and DC antifoam,† had only a suggestive effect on aortic athersclerosis of rabbits(4), but a phenylmethylpolysiloxane‡ altered cholesterol deposition profoundly(5). The present study compares the effect of these 2 silicone fluids in a large number of animals.

Methods and results. In experiments lasting 2 months New Zealand albino rabbits in groups of 6 were fed a stock diet to which 2% cholesterol and .5,% 1%, 2% and 5% of the silicone fluids in weight of diet were added. Weekly serum samples were analyzed for cholesterol by the method of Pearson(6), but the results of the terminal samples only are presented since there were no specific trends in reaching the final value. Tissue cholesterol was determined by the method of Kingsley(7).

The results (Table I) show that the 2 silicone fluids used exert a different biological effect. Addition of phenylmethylpolysiloxane in all concentrations prevents development of severe hypercholesterolemia. The arbitrarily chosen lowest concentration of .5% has as much effect as the 10 times larger amount. The dimethylpolysiloxane did not prevent hypercholesterolemia, except in one experiment where a 1% concentration was used. Even in this instance the effect was less pronounced than that obtained with the other silicone fluid. Cholesterol content of the liver remains esentially unchanged if dimethylpolysiloxane is added to the stock diet containing 2% cholesterol, but increases markedly if the phenylmethylpolysiloxane fluid is fed at the 2% and 5% level. The opposite changes occur in the aorta where the dimethylpolysiloxane produces an increased cholesterol content, whereas addition of phenylmethylpolysiloxane in higher concentrations does not change the cholesterol content of the aorta. The histological lesion of the aorta in a 2% feeding experiment is shown in representative photomicrographs (Fig. 1). Addition of 2% silicone fluids to the stock diet without cholesterol does not alter cholesterol content of the blood serum or of the tissues examined and does not produce histological changes.

Discussion. The cause of the described biological effects of silicone fluids is unknown, therefore the discussion is specula-

* This study was partially supported by a grant from the Dow Corning Center of Aid to Medical Research.

† Supplied by R. R. McGregor, Dow Corning Center of Aid to Medical Research, Midland, Mich.

‡ Coded as XF-10050 by Dow Corning Corp., Midland, Mich.

TABLE I. Cholesterol (g %).

	Serum			Liver			Aorta		
	Avg	S.D.	No.	Avg	S.D.	No.	Avg	S.D.	No.
2% cholesterol	1.373	.454	36	2.497	.701	27	.276	.171	27
Idem + .5% DC 200*	1.015	.334	6	1.867	1.022	5	.543	.342	5
" + 1% DC 200	.689	.314	6	—	—	—	—	—	—
" + 2% DC 200	2.084	.230	6	2.672	.515	6	.682	.366	6
" + 5% DC 200	1.855	.763	6	2.123	1.207	6	.669	.653	6
" + .5% XF-10050†	.559	.240	6	2.786	1.160	3	.557	.554	3
" + 1% XF-10050	.412	.138	6	—	—	—	—	—	—
" + 2% XF-10050	.558	.124	12	5.497	1.995	6	.135	.027	6
" + 5% XF-10050	.481	.156	6	3.589	.782	5	.218	.029	5
Stock diet	.073	.019	30	.218	.044	17	.117	.030	23
Idem + 2% DC 200	.090	.034	24	.175	.032	16	.112	.041	11
" + 2% XF-10050	.072	.013	18	.170	.029	10	.083	.048	10

* Dimethylpolysiloxane. † Phenylmethylpolysiloxane.

tive. The original assumption which led to these investigations that the feeding of silicone fluids may change blood surface tension has not been substantiated. Blood surface tension changed proportionally with cholesterolemia, but not with ingestion of silicone fluids(8).

Comprehensive studies on oral feeding of dimethylpolysiloxane in doses as high as 20 g/kg for one month failed to cause discernible effect in rats(9). However, if cholesterol was added to a diet containing 1% dimethylpolysiloxane renal tubular damage was observed in rabbits(4). The present communi-

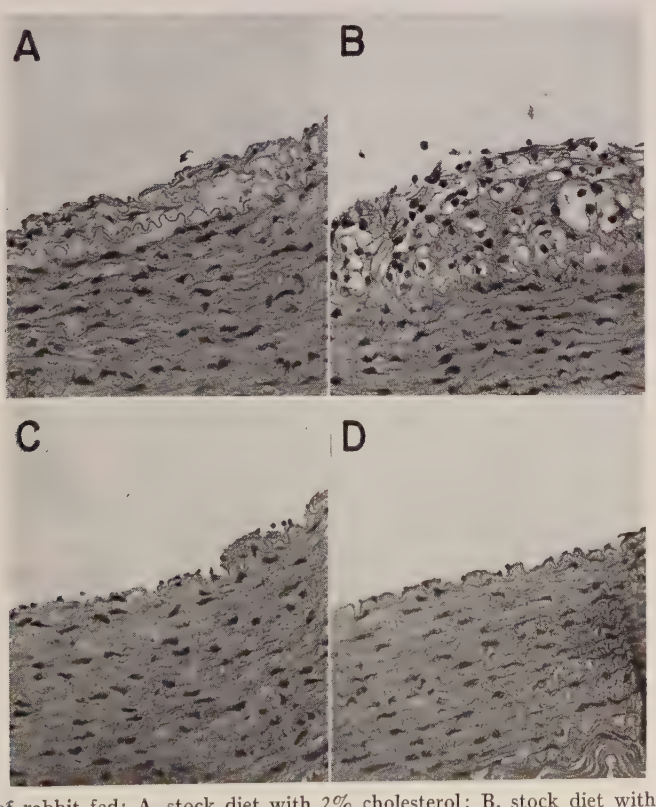


FIG. 1. Aorta of rabbit fed: A, stock diet with 2% cholesterol; B, stock diet with 2% cholesterol and 2% dimethylpolysiloxane; C, stock diet with 2% cholesterol and 2% phenylmethylpolysiloxane; D, stock diet.

cation confirms but does not explain this observation and proliferation of the intima of the aorta containing numerous foam cells (Fig. 1, B) demonstrates the damaging effect of simultaneous feeding of cholesterol and dimethylpolysiloxane. The mechanism by which ingested phenylmethylpolysiloxane prevents severe hypercholesterolemia and the proliferative lesions of the aorta is also obscure, but one may postulate that the phenyl group of this more soluble silicone fluid enhances the transport of triglyceride and cholesterol by the reticuloendothelial system (10).

Summary. Addition of dimethylpolysiloxane to a diet containing 2% cholesterol does not alter hypercholesterolemia and cholesterol content of the liver of rabbits. However, it increases cholesterol content of the aorta and produces atheromatous lesions of the intima. Addition of phenylmethylpolysiloxane to a diet containing 2% cholesterol prevents severe hypercholesterolemia and increases cholesterol content of the liver. It lowers the cholesterol content of the aorta and prevents atheromatous proliferation.

1. Davies, D. F., *Essential Fatty Acids,* 1958, Academic Press, New York.

2. Schwartz, A. M., Terry, J. W., *Surface Active Agents,* 1959, Interscience, London.

3. Kellner, A., Correll, J. W., Ladd, A. T., *J. Exp. Med.,* 1951, v93, 373.

4. Cutting, W. C., *Stanford Med. Bull.,* 1952, v10, 21.

5. Merrill, J. M., Gollan, F., *Circulation,* 1958, v18, 488.

6. Pearson, S., Stern, S., McGaveck, T. H., *J. Clin. Endocrinol.,* 1952, v12, 1245.

7. Kingsley, G. R., Schaffert, R. R., *J. Biol. Chem.,* 1949, v180, 315.

8. Gollan, F., *Fed. Proc.,* 1959, v18, 55.

9. Rowe, V. K., Spencer, H. S., Bass, S. L., *J. Ind. Hyg. and Toxicol.,* 1948, v30, 332.

10. DiLuzio, N. R., *Am. J. Physiol.,* 1959, v196, 884.

Received April 21, 1951. P.S.E.B.M., 1961, v107.

Estrogen-Progesterone Antagonism on Endometrial Carbonic Anhydrase Activity.* (26652)

KAZUKUNI YAMASHITA AND KEN-ICHI KUROUJI (Introduced by G. Pincus)
Department of Physiology, Nagasaki University School of Medicine, Nagasaki, Japan

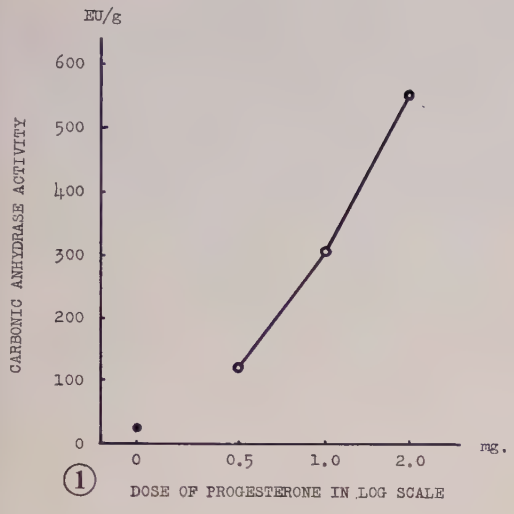
There is ample evidence concerning estrogen-progesterone antagonism in the uterine endometrium. Much has been demonstrated by the histological test based on the proliferative changes in the uterus, the response being rated on the semiquantitative scale. However, little has been done to utilize enzyme determinations in the endometrium as an indicator of hormonal antagonism.

Lutwak-Mann and Adams(1) have studied the effect of stilbestrol on development of progestational activity by the measurements of uterine carbonic anhydrase and progestational proliferation. They observed that in the enzymic test stilbestrol prevented progestational development, and noted that with few exceptions there was good agreement between the enzymic and histological test in evaluating the antagonism.

More recently a work of Miyake and Pincus(2), concerning the antiprogestational activities of estrogens, namely estradiol, estrone, estriol and stilbestrol, in Clauberg rabbits provided quantitative evidence of the antagonism by estimating carbonic anhydrase activity in the uterine endometrium. This was also associated extremely well with proliferative changes (ratio of glandular to total mucosal area). They suggested thereby the superiority of estimation of uterine carbonic anhydrase as a quantitative test for antiprogestational activity.

The present investigation was made to as-

* This investigation was supported by research grant from the Population Council, Inc., Rockefeller Institute. Some hormones were generously supplied by Teikoku Zoki Co. (Japan) through the courtesy of Dr. S. Matsushima.



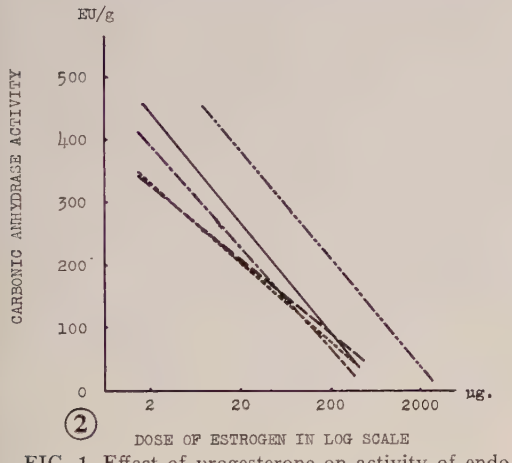


FIG. 1. Effect of progesterone on activity of endometrial carbonic anhydrase in Clauberg rabbits. Progesterone was given in amounts ranging from 0.5 mg to 2.0 mg per animal in 5 divided doses at daily intervals by subcutaneous injection. Carbonic anhydrase activity was expressed as EU/g wet tissue. Five rabbits in each group. ●, control; ○, treated with progesterone.

FIG. 2. Dose-response regression lines of estrogens for endometrial enzymic response to 2.0 mg progesterone per animal. The slope is negative.

——— : Estradiol	$Y = 491 - 174 \cdot \log X$
— — —: Ethinyl estradiol	$Y = 371 - 122 \cdot \log X$
--------: Hexestrol	$Y = 373 - 129 \cdot \log X$
—·—· : Estrone-3-methyl ether	$Y = 441 - 162 \cdot \log X$
——·—· : Estriol-3-methyl ether	$Y = 603 - 172 \cdot \log X$

certain whether the following estrogens are capable of affecting the enzymic response to progesterone in the uterine endometrium; estradiol, ethinyl estradiol, hexestrol, estrone-3-methyl ether and estriol-3-methyl ether.

Material and methods. Immature albino rabbits, approximately 1.5 kg in weight, were used. All of the animals were primed with once daily subcutaneous injections of 5 μg estradiol each for 6 days, according to the Clauberg method(3). The animals were then injected subcutaneously with progesterone and/or test compounds, once daily for 5 days. The test compounds dissolved in sesame oil to graded concentration were used and injection volume was 0.2 cc per animal daily.

The rabbits were sacrificed on the second day after last injection. The uteri were isolated after bleeding thoroughly from the carotid artery. The endometrium was dissected and homogenized with cold distilled water. The homogenate was centrifuged at 2,000 r.p.s. for 10 minutes and the supernatant was used for determination of enzyme activity. The enzyme activity of the extract was assayed colorimetrically by modifying the Philpot and Philpot method(4), one enzyme unit (E.U.) being defined as described in the method of Wilbur and Anderson(5).

$$\frac{To - T}{T} = \text{one unit of activity}$$

where "To" is reaction time without enzyme and "T" is the same with enzyme.

As previously demonstrated by Miyake and Pincus(2), this definition of enzyme unit is also suitable in the colorimetric method of Philpot and Philpot. A linear relationship between (To–T)/T and concentration of enzyme is obtainable in keeping "To" in 60 to 90 seconds and adjusting to get "T" in the range of 10 to 40 seconds.

Results. Activity of endometrial carbonic anhydrase in response to progesterone. In these experiments, estrogen primed rabbits were given amounts of from 0.5 mg to 2.0 mg of progesterone by subcutaneous injection. The results are recorded in Fig. 1. These findings agree with the observations of Lutwak-Mann(6) and Pincus *et al.*(7).

Effects of estrogens on augmented carbonic anhydrase activity caused by progesterone. In experiments to ascertain the antagonistic effect of estrogens, 5 compounds (estradiol, ethinyl estradiol, hexestrol, estrone-3-methyl ether and estriol-3-methyl ether) were employed. Progesterone and estrogen were given simultaneously in 5 divided doses at

TABLE I. Inhibitory Effects of Estrogens on Augmented Activity of Endometrial Carbonic Anhydrase Caused by Progesterone and Percent Inhibition of Progesterone by Estrogen.

Group	Series of hormone	Dose (mg/animal) of Progesterone	Dose (mg/animal) of Estrogen	No. of rabbits	EU/g wet tissue	% inhibition of progesterone by estrogen
1	Control	0	0	5	23 ± 2*	
2	Progesterone	2.0	0	5	554 ± 18	
3	Estradiol + progesterone	2.0	.2	5	89 ± 6	88
			.02	5	270 ± 23	54
			.002	4	436 ± 35	22
4	Ethinyl estradiol + progesterone	2.0	.2	5	110 ± 10	84
			.02	5	174 ± 23	72
			.002	4	358 ± 66	37
5	Hexestrol + progesterone	2.0	.2	5	77 ± 2	90
			.02	5	202 ± 16	66
			.002	5	335 ± 38	41
6	Estrone-3-methyl ether + progesterone	2.0	.2	4	77 ± 13	90
			.02	5	217 ± 24	64
			.002	5	400 ± 47	29
7	Estriol-3-methyl ether + progesterone	2.0	2.0	5	37 ± 3	97
			.2	5	203 ± 34	66
			.02	5	381 ± 28	33
			.002	4	426 ± 36	24

* Mean ± stand. error.

Percent inhibition is expressed as $\frac{Rp - Re}{Rp - C} \times 100$; Rp = uterine enzymic response to progesterone alone; Re = uterine enzymic response to combination of progesterone and estrogen; C = control level.

daily intervals by subcutaneous injection into the neck of the animal. The standard dose of progesterone was 2.0 mg and estrogen doses varied as indicated in Table I.

When estrogens were given in a dose of 0.2 mg along with 2 mg of progesterone, the uterine endometrium had a much decreased carbonic anhydrase activity except in the case of estriol-3-methyl ether (Table I). Percent inhibition of progesterone by each estrogen was 88% by estradiol, 84% by ethinyl estradiol, 90% by hexestrol and estrone-3-methyl ether. These estrogens exhibited almost equal potency in degree of inhibition. However, in the case of estriol-3-methyl ether, enzyme activity was relatively high, an inhibition of 66% being observed. A total of 2.0 mg was necessary for a 97% inhibition of the effect of 2 mg of progesterone.

The dosage response curves (Fig. 2), show the lesser effectiveness of estriol-3-methyl ether; the curves for the 4 other estrogens are quite similar, one to another.

Effect of estrogen on activity of endometrial carbonic anhydrase. Table II presents uterine enzymic responses to these estrogens. Estrogens only were given in a dose of 0.2 mg or 2.0 mg per animal. No significant increase in endometrial carbonic anhydrase above the control was found in any instance. Therefore, it is obvious that estrogen itself is not capable of affecting the activity of endometrial carbonic anhydrase.

Discussion. These results show that estrogens we have used can prevent the augmented activity of uterine carbonic anhy-

TABLE II. Effects of Estrogens on Carbonic Anhydrase Activity of Uterine Endometrium.

Series of estrogen	Dose (mg/animal)	No. of rabbits	EU/g (wet tissue)
Control	.2	5	23 ± 2
Estradiol	.2	5	32 ± 8
Ethinyl estradiol	.2	3	27 ± 4
Hexestrol	.2	5	34 ± 9
Estrone-3-methyl ether	.2	4	32 ± 7
Estriol-3-methyl ether	2.0	4	33 ± 8

Results expressed as mean ± stand. error. Each estrogen was injected subcut. in 5 divided doses at daily intervals.

drase caused by progesterone. It seems that the antiprogestational ability of estrogen is almost the same in estradiol, ethinyl estradiol, hexestrol and estrone-3-methyl ether, but approximately 1/10 of the others in estriol-3-methyl ether, if given systemically. From the data, a linear log dose response relation of 2 antagonistic hormones was obtained in amounts ranging from 0.002 mg to 0.2 mg of estradiol, ethinyl estradiol, hexestrol and estrone-3-methyl ether and in doses of 0.02-2.0 mg in estriol-3-methyl ether, the regression about the line being highly significant. Thereby, quantitative differences in the potency of these estrogens to prevent progestational development may be provided.

Previously, Miyake and Pincus(2) showed that other estrogens (estradiol, estriol, estrone and stilbestrol) suppressed the enzymic response to progesterone, the extent of which correlated extremely well with the degree of pseudo-pregnant proliferation. The antiprogestational activities of these estrogens were all approximately the same. In their experiments, almost the same procedure as that reported here was employed. The response regression line of estradiol on progestational development is in satisfactory agreement with our data.

Considering that estrone and estriol were shown, according to them, to be the same in antagonistic potency, it would be expected that inhibitory abilities of estrone- and estriol-3-methyl ether would be the same because of certain similarities in chemical properties. However, this is not the case in our experiments. The results indicate a marked difference in the abilities of estrone- and estriol-3-methyl ether to affect the action of progesterone. Estriol-3-methyl ether seems to have approximately 1/10 the potency of estrone-3-methyl ether if the comparison is made from our regression line.

The authors are indebted to Professor T. Suzuki for many helpful suggestions and to Dr. K. Hirai for assistance throughout this study. We also wish to thank Dr. G. Pincus, Worcester Foundation for Experimental Biology, and Dr. W. O. Nelson, Population Council, Inc., Rockefeller Institute, for kindly supporting this work.

1. Lutwak-Mann, C., Adams, C. E., *J. Endocrinol.*, 1957, v15, 43.

2. Miyake, T., Pincus, G., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 478.

3. Clauberg, C., *Zentr. Gynäkol.*, 1930, v54, 2757.

4 Philpot, F. J., Philpot, J. St. L., *Biochem. J.*, 1936, v30, 2191.

5. Wilbur, K. M., Anderson, N. G., *J. Biol. Chem.*, 1948, v176, 147,

6. Lutwak-Mann, C., *J. Endocrinol.*, 1955, v13, 26.

7. Pincus, G., Miyake, T., Merrill, A. P., Longo, P., *Endocrinology*, 1957, v61, 528.

Received April 24, 1961. P.S.E.B.M., 1961, v107.

Inhibitory Effect of Ammonium Ions on Influenza Virus in Tissue Culture. (26653)

E. M. JENSEN, E. E. FORCE AND J. B. UNGER (Introduced by O. C. Liu)
Research and Development Division, Smith Kline and French Laboratories, Philadelphia, Pa.

While examining a series of pyrimidines for antiviral activity in a tissue culture system, it was observed that one compound exhibited a marked inhibition of the cytopathic effect (CPE) of influenza virus. Subsequent studies with other preparations of the same compound showed varying degrees of activity, and in some cases a complete lack of activity. This inhibitory effect was eventually shown to be due entirely to trace amounts of ammonium ion present in the active preparations as an impurity. Further studies have shown that comparable results can be obtained with a number of inorganic and organic ammonium salts. This report describes in detail the virus inhibition and discusses possible explanations for the observed effect. It is also shown that under normal growth conditions sufficient ammonia may be accumulated in the medium to pro-

TABLE I. Inhibition of Viral CPE by Several Ammonium Salts.

Compound	CPE at μg/ml of compound*								Minimum effective level	
	512	256	128	64	32	16	8	4	μg/ml	μmoles of ammonia
Ammonium chloride	Toxic	0	0	0	0	2+	3+	4+	16	.30
" sulfate	"	Toxic	0	0	2+	4+	4+	4+	32	.49
" citrate	"	0	2+	3+	4+	4+	4+	4+	128	1.08

* All tubes inoculated with 1000 $TCID_{50}$'s/ml influenza virus, PR-8.

tect the cells from influenza virus.

Materials and methods. The virus employed was Influenza A, strain PR-8, which was in its 126th passage in embryonated chicken eggs *via* the allantoic route. Virus dilutions were prepared in tissue culture maintenance medium described below. Infectivity titrations of virus were performed both in chick embryos and in tissue culture and were calculated by the method of Reed and Muench(1). Hemagglutination (HA) titrations were conducted by the method of Salk(2) and are expressed as the reciprocal of the dilutions.

The cell culture employed in this study was a stable line of epithelium-like cells originally isolated from a dog kidney, which has undergone 76 subpassages in our laboratory. The growth medium for these cells consisted of Eagle's basal medium(3) supplemented with 10% calf serum. Maintenance medium consisted of Eagle's basal medium supplemented with 2.5% calf serum.

Virus inhibition studies in tubes were conducted by inoculating 2 series of duplicate tissue culture tubes with 2-fold dilutions of the test compound ranging from 1024 μg/ml to 4 μg/ml. One series was retained as a toxicity control and the other series was inoculated with 1000 $TCID_{50}$'s of influenza virus, strain PR-8. Generally the compound was added one hour before addition of the virus. The results, observed 48 or 72 hours after infection are expressed in terms of CPE, graded on a scale of 0 for no cellular degeneration to 4+ for complete destruction of cells.

Ammonia assays were conducted by the method of Conway(4) and colorimetric determinations of the Nesslerized samples were made with a Bausch and Lomb Spectronic "20" colorimeter at 500 mμ.

Results. Table I shows the inhibitory effect of ammonium ions on the CPE of influenza virus. The results show that viral inhibition is roughly proportional to the actual amount of ammonia present in each salt. The lack of perfect correlation may be due to the limits of accuracy of the test system and to the difference in dissociation constants of the salts used. The possibility of the anions being inhibitory can be excluded by the fact that normal tissue culture medium contains approximately a thousand-fold greater concentration of chloride ion than is added in the form of ammonium chloride.

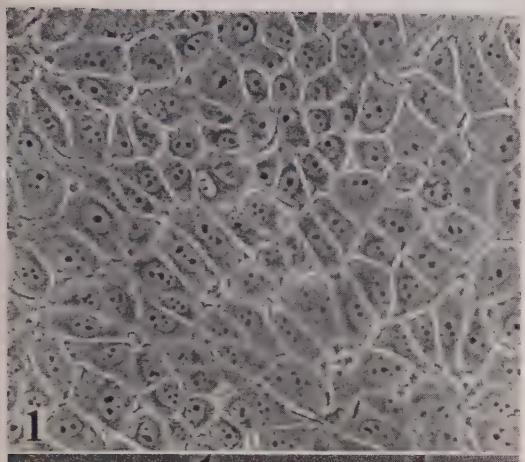
The degree of protection afforded by ammonium ion is shown in Fig. 1-3. These 3 photographs of unstained dog kidney monolayer cultures show clearly that 40 μg/ml of NH_4Cl is sufficient completely to protect the cells from an otherwise lethal inoculum of influenza virus. It is further shown that 40 μg/ml of NH_4Cl has no apparent effect upon the cell layer. The protection was obtained only when the salt was added either before or simultaneously with the infection. A delay of one hour or more resulted in very little protection.

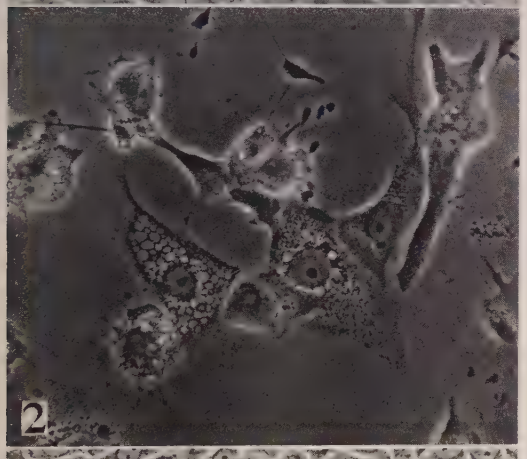
The protection observed as a function of CPE was also observed as a suppression of viral synthesis. Table II shows the inhibitory effect of 40 μg/ml of NH_4Cl as measured by CPE, hemagglutination and infectivity titers. The tissue cultures were infected one hour after addition of the NH_4Cl and

TABLE II. Inhibition of Viral Synthesis by 40 μg/ml of NH_4Cl.

NH_4Cl	Inoculum* $TCID_{50}$/ml	CPE	HA/ml	EID_{50}/ml
—	1000	3+	4096	$10^{5.0}$
40 μg/ml	"	0	<4	$10^{3.7}$

* Influenza virus, PR-8.





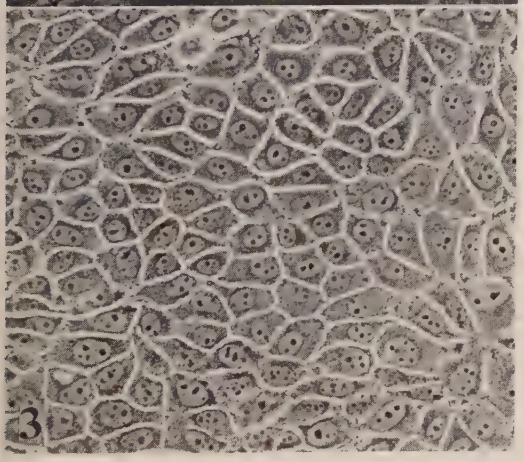


FIG. 1. Normal monolayer culture of dog kidney cells. (Phase contrast, magnif. 269 ×.)

FIG. 2. Dog kidney cell culture similar to Fig. 1 48 hr after infection with 1000 $TCID_{50}$'s of influenza virus, PR-8. Majority of the cells have been destroyed and dislodged from cover slip. Remaining cells show severe viral CPE. (Phase contrast, magnif. 336 ×.)

results were observed at 48 hours.

Preliminary attempts to establish the mechanism of action of the inhibition have been unsuccessful. Ammonium chloride is not virucidal at concentrations substantially higher than those required for inhibition. As much as 1000 μg/ml of NH_4Cl mixed with 10^5 EID_{50}'s of influenza virus in maintenance medium caused no reduction in chick embryo infectivity titers when incubated for 5 hours at 37°C. Furthermore, the effect cannot be attributed to a pH change. Addition of 1000 μg/ml of NH_4Cl to maintenance medium lowers the pH by less than 0.1 of a pH unit; a change substantially less than those encountered in normal tissue culture growth.

Table III shows the results of an experiment designed to determine the effect of ammonium ions on adsorption of influenza virus to host cells. Cultures 2 and 4 were treated with 40 μg/ml of NH_4Cl. One hour later all cultures were infected with 1000 $TCID_{50}$'s of influenza virus. Cultures 1 and 2 were incubated for 48 hours, whereas the remaining cultures were washed free of virus and NH_4Cl one hour after infection, refed and incubated for 48 hours. The results show that whether ammonium ion was present or not, the virus was adsorbed and possibly absorbed in a period of less than one hour. Protection of the cells from the virus was obtained only if the ammonium ion was left in contact with the cells for the full incubation period. This experiment shows that the virus inhibition was not due to an interference with virus adsorption and this conclusion was also supported by the observation that as much as 10,000 μg/ml of NH_4Cl did not interfere with influenza hemagglutination titrations.

During this work it was observed that actively growing cells released measurable amounts of ammonia into the medium and that after several days this reached levels which should be inhibitory to virus growth. As expected, it was shown that 3-day-old cul-

FIG. 3. Monolayer dog kidney cell cultures showing protective effect of ammonium ions. Culture infected same as that in Fig. 2 but culture medium contained 40 μg/ml of NH_4Cl. (Phase contrast, magnif. 269 ×.)

TABLE III. Effect of Ammonium Ions on Adsorption of Influenza Virus to Dog Kidney Cells.

Culture No.	NH_4Cl (μg/ml)	Virus inoculum* ($TCID_{50}$/ml)	Treatment of tissue culture	48 hr results CPE	48 hr results HA/ml
1	—	1000	Not washed or refed	4+	2048
2	40	"	*Idem*	0	<4
3	—	"	Washed and refed 1 hr after infection	4+	4096
4	40	"	*Idem*	3-4+	2048

* Influenza virus, PR-8.

tures could not be infected if the medium was not first replaced with fresh maintenance medium. The following experiment was conducted to determine whether this non-susceptibility of old cultures could be attributed to the ammonia.

Maintenance medium was collected from several T-30 flask cultures after 3 days exposure to the cells. The medium was divided into 2 portions, one of which was saved unchanged. The other portion was adjusted to pH 10 with 1 N · NaOH and lyophilized to remove the ammonia. It was then reconstituted to its original volume with sterile distilled water and neutralized with 1 N · HCl. Three healthy T-30 flask cultures were then arranged as shown in Table IV. Culture 1 was refed with the untreated old medium. Culture 2 was refed with the lyophilized reconstituted medium (ammonia partially removed) and culture 3 was refed with fresh maintenance medium. All cultures were then infected with 1000 $TCID_{50}$'s of influenza virus and all 3 cultures were incubated at 37°C for 48 hours, at which time they were observed for CPE and the fluids were titrated for hemagglutinins and chick embryo infectivity. The medium from each culture was assayed for total free ammonia at time of infection.

The results given in Table IV show that although some viral synthesis occurred in the cells refed with untreated old medium, the yield was only a fraction of that produced in cells refed with old medium from which the ammonia had been partially removed, or with new medium. The viral effect in culture 1 was not apparent as a CPE; however, the cells did not appear as healthy as the control cultures, undoubtedly because of lack of nutrients and/or accumulation of other waste products. This probably also accounts for the difference in virus titers in cultures 2 and 3. Although cultures 2 and 3 were not free of ammonia at zero-time, the level was apparently not high enough completely to suppress virus production.

Discussion. The virus-host cell system employed in this study appears similar to systems reported by other workers(5,6) in that a large initial inoculum of virus is necessary for development of CPE. The infection results in a high yield of incomplete hemagglutinating virus but a very low yield of infective virus. Subpassage of the virus in this system results in its eventual disappearance.

The demonstration that a simple inorganic cation such as ammonia can cause a marked inhibition of viral synthesis and prevent subsequent destruction of the host cell is of considerable interest. As shown here, chemotherapeutic studies may yield misleading results due to trace amounts of ammonia present in the test compounds. It is conceivable also that some of the undefined inhibitors of viruses found in various organic materials,

TABLE IV. Inhibitory Effect of Old Tissue Culture Medium on Virus Synthesis and Its Relation to Ammonia Content.

Culture No.	Medium	CPE	HA/ml	EID_{50}
1	Old medium (22 μg NH_4^+/ml)*	0	32	$10^{4.66}$
2	Old medium - ammonia partially removed (9 μg NH_4^+/ml)	3+	256	$10^{5.33}$
3	New medium (11 μg NH_4^+/ml)*	3+	2048	$10^{6.5}$

All cultures inoculated with 1000 $TCID_{50}$'s influenza virus and results read at 48 hr.

* Concentration of ammonia present at time of infection.

for example that of lactalbumin hydrolysate (7), may be due to presence of ammonia. This may also account for the apparent variations observed in susceptibility of tissue culture cell systems to virus infections. Since ammonia tends to accumulate in the medium during cell growth, the age of culture media becomes a significant factor in cell susceptibility to infection. Preliminary studies indicate that the primary source of ammonia in fresh medium is glutamine. Inasmuch as glutamine has been shown to be required for virus synthesis in some systems(8) and markedly to change the type of CPE observed in others(9) careful consideration must be given to incorporation or exclusion of this material in all systems.

Of particular interest is the mechanism by which this inhibition is effected. Since addition of the ammonium salts must be made prior to or simultaneously with the virus inoculum for maximum protection, one would suspect an interference with virus adsorption to the host cell or a direct virucidal effect. Experimental evidence seems to rule out these explanations and consequently some intracellular mechanism must be suspected. An understanding of this inhibition may shed new light on the process of virus replication with possible applications to viral chemotherapy. Studies are now in progress to explore the mechanism involved as well as the effect of ammonium ions on other virus-cell systems. The effect of various other inorganic ions and the smaller organic amines is also being investigated.

Summary. Ammonium ions in trace amounts were shown to exert a very marked protective effect upon a tissue culture system infected with influenza virus. The ions inhibited viral synthesis as well as viral CPE and the effect was apparently not due to interference with virus adsorption or to a direct inactivation of the virus. It was also shown that the tissue culture system produced sufficient ammonia during growth to render itself insusceptible to the virus.

Addendum. Since the preparation of this manuscript a paper has appeared, Eaton, M. D. and Scala, A. R., *Virology,* 1961, v13, 300, in which inhibition of both influenza and Newcastle disease viruses by ammonia in Krebs 2 cells was reported. The results and conclusions given by these authors are very similar to those presented here.

1. Reed, L. J., Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

2. Salk, J. E., *J. Immunol.,* 1944, v49, 87.

3. Eagle, H., Oyama, V. I., Levy, M., Freeman, A. E., *J. Biol. Chem.,* 1957, v226, 191.

4. Conway, E. J., Byrne, A., *Biochem. J.,* 1933, v27, 419.

5. Henle, G., Girardi, A., Henle, W., *J. Exp. Med.,* 1955, v101, 25.

6. Mason, E. J., Kaufman, N., *J. Inf. Dis.,* 1960, v107, 245.

7. Holper, J. C., Marquis, G. S., Jr., *ibid.,* 1959, v105, 288.

8. Eagle, H., Habel, K., *J. Exp. Med.,* 1956, v104, 271.

9. Reissig, M., Black, F. L., Melnick, J. L., *Virology,* 1956, v2, 836.

Received April 27, 1961. P.S.E.B.M., 1961, v107.

Structure-Activity Relationships of Adrenocorticoids. (26654)

I. RINGLER, S. MAUER AND E. HEYDER (Introduced by Earl H. Dearborn)

Experimental Therapeutics Research Section, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

The past 8 years have heralded significant advances in syntheses of cortisone and hydrocortisone analogs possessing quantitative and qualitative differences in metabolic activities. Though it is not possible to predict accurately the qualitative metabolic effects of chemical substituents on hydrocortisone, this communication describes the influence of 1,2 unsaturation, 6α-methylation, 9α-fluorination, 16α-hydroxylation and 16α,17α-ketalization, alone and in combination, on liver glycogen deposition and thymus involution in

TABLE I. Comparative Adrenocorticoid Activities.

Steroid	No. of assays	Relative potency (95% confidence limits)* Liver glycogen deposition	Thymus involution
Hydrocortisone		1.0	1.0
16α-hydroxyhydrocortisone	4	.4 (.3- .6)	.3 (.2- .3)
Hydrocortisone-16α,17α-acetonide	3	2.0 (1.6- 2.5)	2.2 (2.0- 2.5)
Δ^1-hydrocortisone	2	4.1 (2.9- 5.7)	2.3 (1.7- 3.2)
Δ^1-16α-hydroxyhydrocortisone	1	.9 (.5- 1.0)	1.3 (.8- 2.1)
Δ^1-hydrocortisone-16α,17α-acetonide	1	13.0 (7 -22)	17.0 (10 -28)
6α-methylhydrocortisone†	1	9.5 (6.2-14.5)	5.8 (4.5- 7.7)
6α-methyl-16α-hydroxyhydrocortisone	2	1.1 (.8- 1.5)	1.2 (1.0- 1.5)
6α-methylhydrocortisone-16α,17α-acetonide	1	10.0 (5 -18)	14.0 (10 -20)
9α-fluorohydrocortisone	2	6.1 (4.8- 7.6)	5.6 (4.5- 6.9)
9α-fluoro-16α-hydroxyhydrocortisone	2	7.9 (5.5-11.5)	2.4 (1.7- 3.2)
9α-fluorohydrocortisone-16α,17α-acetonide	2	14.0 (10 -20)	14.0 (12 -17)
6α-methyl-9α-fluorohydrocortisone†	2	9.0 (6.8-11.9)	9.8 (8.0-11.9)
6α-methyl-9α-fluoro-16α-hydroxyhydrocortisone	3	2.8 (2.3- 3.4)	4.2 (3.6- 4.8)
6α-methyl-9α-fluorohydrocortisone-16α,17α-acetonide	1	15.0 (9 -24)	29.0 (21 -40)
Δ^1-6α-methylhydrocortisone	2	8.4 (5.3-10.4)	10.0 (8 -13)
Δ^1-6α-methyl-16α-hydroxyhydrocortisone	2	2.0 (1.2- 3.4)	2.2 (1.8- 2.6)
Δ^1-6α-methylhydrocortisone-16α,17α-acetonide	2	24.0 (19 -29)	28.0 (22 -34)
Δ^1-9α-fluorohydrocortisone	2	13.0 (8 -22)	16.0 (13 -20)
Δ^1-9α-fluoro-16α-hydroxyhydrocortisone	9	6.1 (5.5- 6.7)	3.9 (3.6- 4.3)
Δ^1-9α-fluorohydrocortisone-16α,17α-acetonide	5	32.0 (16 -39)	33.0 (29 -38)
Δ^1-6α-methyl-9α-fluorohydrocortisone‡	2	16.0 (13 -20)	25.0 (20 -31)
Δ^1-6α-methyl-9α-fluoro-16α-hydroxyhydrocortisone	4	4.9 (4.1- 5.9)	5.5 (4.8- 6.3)
Δ^1-6α-methyl-9α-fluorohydrocortisone-16α,17α-acetonide	3	21.0 (18 -24)	70.0 (60 -81)

* Relative potency × hydrocortisone. † Steroids kindly supplied by Upjohn Co., Kalamazoo, Mich. ‡ 21-acetate.

an attempt to quantitate the increments and decrements in biological activity induced by these chemical modifications.

Materials and methods. In a 5-day bioassay, liver glycogen deposition and thymus involution were simultaneously determined in adrenalectomized, immature (40-60 g Sherman strain) male rats. Animals were maintained with Purina Lab Chow and 1% NaCl drinking fluid *ad lib.* Six concentrations of test steroids were assayed in parallel with 6 concentrations of hydrocortisone, using 4 rats per group. Twenty-four hours after adrenalectomy the rats were injected subcutaneously with steroid suspended in 0.2 ml of carboxymethyl-cellulose vehicle(1), excluding benzyl alcohol. Control animals received vehicle only. Steroid was administered daily for an additional 4 days. The rats were fasted 15 hours before and 7 hours after last injection to insure maximum depletion of liver glycogen. Animals were then anesthetized with sodium pentobarbital and livers quickly excised, weighed and hydrolyzed in 30% potassium hydroxide(2). Thymi were removed and weighed on Roller-Smith torsion balance. Liver glycogen was measured by the anthrone method of Seifter *et al.*(3). Data were statistically analyzed by fitting straight lines by the method of least squares and computing the corresponding equation. Potency estimates were combined by the method of Wilcoxon and Haynes (unpublished).

Results. Modification of the hydrocortisone molecule by either introduction of a double bond at the 1,2 positions, 9α-fluoro, 6α-methyl, 16α-hydroxy or 16α,17α-isopropylidenedioxy, or combinations of these structural constituents endows the steroid with distinct biological potencies (Table I). For the majority of the steroids studied, the relative potencies calculated for liver glycogen deposition and thymus involution compared favorably, suggesting that potency estimates from various assays of glucocorticoid

activity would be similar if conducted simultaneously. This is by no means a new concept, but has been advocated by Stephenson (4). These potency estimates have been tabulated to show the activity-enhancing capacity of each substituent on both liver glycogen deposition and thymus involution (Table II).

The glycogenic activity of hydrocortisone was increased approximately 2-fold by introduction of a double bond at positions 1,2. This augmentation in activity represents the

TABLE II. Action of Functional Groups on Adrenocorticoid Potency.

Parent steroid (Potency = 1)	Substituent									
	Δ^1		6α-methyl-		9α-fluoro-		16α-hydroxy-		16α,17α-acetonide-	
	Biological potency relative to parent steroid									
	LGD	TI	LGD	TI	LGD	TI	LGD	TI	LGD	TI
Hydrocortisone	4.1	2.3	9.5	5.8	6.1	5.6	.4	.3	2.0	2.2
16α-hydroxylhydrocortisone	2.2	4.3	2.7	4.0	—	8.0	—	—	—	—
Hydrocortisone, 16α,17α-acetonide	6.5	7.7	5.0	6.4	7.0	6.4	—	—	—	—
Δ^1-hydrocortisone	—	—	2.0	4.3	3.2	6.9	.2	.6	3.2	7.4
Δ^1-16α-hydroxyhydrocortisone	—	—	2.2	1.7	6.8	3.0	—	—	—	—
Δ^1-hydrocortisone-16α,17α-acetonide	—	—	1.9	1.7	2.5	1.9	—	—	—	—
6α-methylhydrocortisone	.9	1.8	—	—	1.0	1.7	.1	.2	1.1	2.4
6α-methyl-16α-hydroxyhydrocortisone	1.8	1.8	—	—	2.5	3.5	—	—	—	—
6α-methylhydrocortisone-16α,17α-acetomide	2.5	2.1	—	—	1.5	2.1	—	—	—	—
9α-fluorohydrocortisone	2.1	2.9	1.5	1.8	—	—	1.3	.4	2.3	2.5
9α-fluoro-16α-hydroxyhydrocortisone	.8	1.6	.4	1.8	—	—	—	—	—	—
9α-fluorohydrocortisone-16α,17α-acetonide	2.3	2.4	1.1	2.1	—	—	—	—	—	—
6α-methyl-9α-fluorohydrocortisone	1.8	2.6	—	—	—	—	.3	.4	1.7	2.9
6α-methyl-9α-fluoro-16α-hydroxyhydrocortisone	1.7	1.3	—	—	—	—	—	—	—	—
6α-methyl-9α-fluorohydrocortisone-16α,17α-acetonide	1.4	2.4	—	—	—	—	—	—	—	—
Δ^1-6α-methylhydrocortisone	—	—	—	—	1.9*	2.5*	.2	.2	2.9	2.9
Δ^1-6α-methyl-16α-hydroxyhydrocortisone	—	—	—	—	2.5	2.5	—	—	—	—
Δ^1-6α-methylhydrocortisone-16α,17α-acetonide	—	—	—	—	.8	2.5	—	—	—	—
Δ^1-9α-fluorohydrocortisone	—	—	1.2*	1.6*	—	—	.5	.2	2.5	2.1
Δ^1-9α-fluoro-16α-hydroxyhydrocortisone	—	—	.8	1.4	—	—	—	—	—	—
Δ^1-9α-fluorohydrocortisone-16α,17α-acetonide	—	—	.7	2.1	—	—	—	—	—	—
Δ^1-6α-methyl-9α-fluorohydrocortisone	—	—	—	—	—	—	.3	.2	1.3	2.8
Mean	2.3	2.8	2.4	2.9	3.3	3.9	.4	.3	2.1	3.2

LGD = liver glycogen deposition; TI = thymus involution.

* Acetate.

mean increment for 12 steroids with ranges from 0.8 to 6.5. Substitution of a methyl group in the α position at carbon 6 enhanced the ability of parent steroids to induce glycogen deposition approximately 2-fold (range 0.4-9.5). Fluorination at carbon 9 resulted in a 3-fold increase in activity (range 0.8-7.0). Although the addition of a hydroxyl group at the 16α position decreased the glycogenic potencies of the various corticoids studied by approximately 0.4 (range 0.1-1.3), formation of the 16α,17α-isopropylidenedioxy derivatives increased it some 2-fold (range 1.1-3.2).

The thymolytic potency of the steroids included in this study was increased in all cases by introduction of a double bond in positions 1,2, 6α-methyl, 9α-fluoro and the 16α,17α-acetonide. Data in Table II demonstrate that an α hydroxyl group at carbon 16 depressed the action of corticosteroids by one-third.

Discussion. Though not conclusive, these data suggest a possible means of predicting the effects of structural modifications on the biological activities of corticosteroids. Thus, introduction of a double bond at positions 1, 2 or 6α-methylation, or 16α,17α-ketalization increases glycogenic and thymolytic activity 2- to 3-fold. The presence of a 9α-fluoro group enhances activity 3 to 4 times. 16α Hydroxylated steroids were 0.3 to 0.4 times as efficacious as the nonhydroxylated parent steroids.

In comparing increments of biological activity, one has the impression that they are greater for either Δ^1, 6α-methyl or 9α-fluoro substituents on hydrocortisone and 16α-hydroxy hydrocortisone than on more complex substituted steroids. It is thus apparent that modification in biological activity induced by a specific permutation of the steroid is generally dependent on presence of other activity-influencing groups.

Presentation of enhancement factors for numerous functional groups has previously been outlined by Fried and Borman(5). They have tabulated a series of enhancement factors, which, when multiplied by the biological activity of the parent steroid, results in a numerical figure indicative of activity of the substituted steroid. Some of these enhancement factors(5) are presented in Table III with those recorded in this publication.

TABLE III. Adrenocorticoid Activity-Enhancement Factors.

Functional group	Liver glycogen deposition	Thymus involution	Anti-inflammatory
9α-Fluoro	10 * 3.3	3.9	7–10*
1 Dehydro	3–4 * 2.3	2.8	3–4 *
6α-Methyl	2–3 * 2.4	2.9	1–2 *
16α-Hydroxyl	.4–.5* .4	.3	.1–.2*

* Fried and Borman(5).

With the exception of the enhancement factor for the 9α-fluoro substituent, the data are in agreement.

Application of the indicated enhancement factors to steroids not possessing either 11β, 17α or 21-hydroxyl functions has not been fully studied. However, it has been recognized in this laboratory that ketalization of the 16α,17α-hydroxy groups of 11-keto adrenocorticoids does not potentiate biological activity.

Summary. The influence of 1,2 unsaturation, 6α-methylation, 9α-fluorination, 16α-hydroxylation, and 16α,17α-ketalization alone and in combination on glycogenic and thymolytic activity has been simultaneously assessed in immature adrenalectomized rats. Good agreement between glycogenic and thymolytic relative potencies was observed. Unsaturation at positions 1,2 or 6α-methylation, or 16α,17α-ketalization increased glycogenic and thymolytic activity 2- to 3-fold. Presence of a 9α-fluoro group enhanced activity 3- to 4-fold. 16α-Hydroxylation reduced activity by $\frac{1}{3}$ to $\frac{1}{4}$.

The authors are indebted to W. Pierce, E. Ryerson, W. Cook and R. Steinhoff for technical assistance; to J. D. Haynes for statistical analyses; and to the Organic and Biochemical Research Sections for generous supplies of steroids.

1. Stafford, R. O., Barnes, L. E., Bowman, B. J., Meinzinger, M. M., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 371.

2. Pabst, M. L., Sheppard, R., Kuizenga, M. H., *Endocrinology,* 1947, v41, 55.

3. Seifter, S., Dayton, S., Novic, B., Muntwyler, E., *Arch. Biochem.,* 1950, v25, 191.

4. Stephenson, N. R., *J. Pharm. and Pharmacol.*, 1960, v12, 411.
5. Fried, J., Borman, A., *Vitamins and Hormones*, Acad. Press, 1958, vXVI, 303.

Received April 28, 1961. P.S.E.B.M., 1961, v107.

Activity of α-Phenoxyalkyl Penicillins Against Sensitive and Resistant Staphylococci. (26655)

A. GOUREVITCH, G. A. HUNT, J. R. LUTTINGER, C. C. CARMACK, AND J. LEIN
(Introduced by H. A. Feldman)
Research Division, Bristol Laboratories, Syracuse, N. Y.

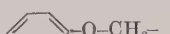
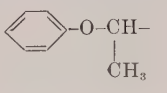
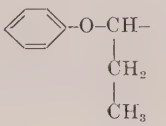
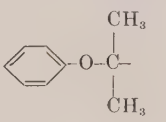
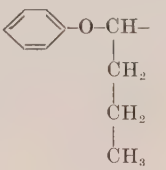
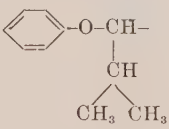
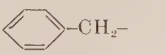
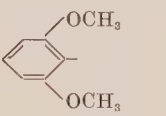
While studying the antimicrobial activity of a large number of synthetic penicillins, it became apparent that members of the α-phenoxyalkyl penicillin series were displaying significant activity against penicillinase-producing resistant staphylococci. Preliminary microbiological data and methods of synthesis of a number of such penicillins have been reported(1,2). A detailed study was done on certain members of this series of penicillins using both sensitive and resistant staphylococci in an attempt to delineate the extent of the activity and relate some of the properties of the penicillins to the structure. Comparisons of these penicillins were made with penicillin G and dimethoxyphenyl penicillin, the former being penicillinase sensitive and the latter extremely penicillinase resistant (3). Studies were also carried out to determine the *in vivo* effectiveness of the different penicillins in curing penicillin-sensitive and penicillin-resistant *Staphylococcus aureus* infections in mice.

Materials and methods. The penicillins used in this study were alkyl derivatives of phenoxymethyl penicillin substituted in the α position. The side chains of the penicillins studied are shown in Table I together with the side chains of controls, benzylpenicillin (penicillin G) and dimethoxyphenyl penicillin. The last was used as the monohydrate of the sodium salt. All the other penicillins were potassium salts. Benzylpenicillin (penicillin G), α-phenoxyethyl penicillin (phenethicillin), dimethoxyphenyl penicillin, and phenoxymethyl penicillin (penicillin V) were obtained from commercial sources, the first 3 being Bristol Laboratories production lots, the last one a Lilly product. The other penicillins were synthesized by the Organic Chemistry Dept. of Bristol Laboratories.

Cultures were maintained on porcelain beads(4), on nutrient agar slants, or frozen in milk suspension. The inoculum used for determination of the minimum inhibitory concentration was prepared by overnight incubation of a bead or a loopful from a slant culture in heart infusion broth. Standard 2-fold serial dilution technics in heart infusion broth (Difco) or in heart infusion mixed 1:1 with pooled filter-sterilized human serum were used. Usually, the overnight culture was diluted 10^4, but other dilutions were also used as noted below. Cultures for *in vivo* challenges were grown overnight in veal infusion broth. The challenge dose of *Staphylococcus aureus,* Smith strain, consisted of a dose of organism equivalent to 100 LD_{50} or approximately 8×10^5 viable cells per mouse, suspended in 5% hog gastric mucin. The challenge dose of *S. aureus,* 1633-2 strain, consisted of a dose of organism equivalent to 50 LD_{50} or approximately 1.1×10^8 viable cells per mouse prepared similarly. This latter strain was isolated from clinical material and is a penicillinase producer.

Penicillinase inactivation of the penicillins was determined by the method of Henry and Housewright(5). Acetone-ether treated staphylococcal cells prepared according to Gilson and Parker(6) from cells of the 52-75 strain of *Staphylococcus aureus* were used as the source of penicillinase. The culture was induced for 3 hours at 28°C on a rotary shaker with 100 μg/ml of benzylpenicillin added at 0 time, followed by a similar addi-

TABLE I. Side Chains of Penicillins.

Penicillin	Structure
Phenoxymethyl penicillin	
α-Phenoxyethyl penicillin	
α-Phenoxypropyl penicillin	
α-Phenoxyisopropyl penicillin	
α-Phenoxybutyl penicillin	
α-Phenoxyisobutyl penicillin	
Controls:	
Benzylpenicillin	
Dimethoxyphenyl penicillin	

tion at 45 minutes and by 100 μg/ml of dimethoxyphenyl penicillin at 90 minutes. The dry preparation was stored in the refrigerator. For determination of penicillin inactivation, a suspension of cells was prepared in 0.017 M bicarbonate buffer and homogenized with teflon grinders. To the main compartment of a Warburg respirometer, 2.7 ml of this penicillinase preparation were added and 0.5 ml of penicillin solution in the same buffer was placed in the side arm. The amount of enzyme preparation in the vessel depended on the penicillinase susceptibility of the penicillin. For most penicillins, 405 μg per vessel was used. For more resistant penicillins, a larger amount was used. The vessels were equilibrated with a gas mixture containing 95% oxygen and 5% carbon dioxide. In all cases, the penicillins were present in 0.01 molar solution. Determinations were run in duplicate, and expressed in cu mm of CO_2 per mg dry material per hour. Benzylpenicillin was always used as a control and final calculations were made in per cent of the rate obtained with penicillin G.

Median curative doses (CD_{50}) were determined using acute infections produced by intraperitoneal injection of challenge doses of the organisms. The challenge was immediately followed by intramuscular treatment with graded doses of aqueous solutions of the various penicillins. The CD_{50} was determined from a probit transformation of percentage of deaths at each dose level using graphic estimation of the 50% point.

Results. In vitro *experiments.* The minimum inhibitory concentrations (MIC) of various penicillins against penicillin-sensitive (Smith) and penicillin-resistant (1633-2) strains of *S. aureus* in presence and in absence of serum are presented in Table II. Examination of the data reveals that the activity against the Smith strain decreases as the alkyl group becomes larger. This relationship holds both in absence and presence of 50% human serum. In the case of the 1633-2 strain which is penicillin G resistant, a greater activity is seen in the case of some of the higher homologs. Thus the α-phenoxypropyl and α-phenoxyisobutyl penicillins appear to be particularly active. The presence of 50% serum, however, alters these relationships somewhat, persumably due to different degrees of serum binding. Comparison of these results with that of dimethoxyphenyl penicillin shows that while this penicillin is not very active, the MIC's obtained with the resistant strain are very similar to that obtained with the sensitive strain. Also, the presence of serum had essentially no effect on

TABLE II. Minimum Inhibitory Concentrations (MIC) of α-Phenoxyalkyl Penicillins against Penicillin Sensitive (Smith) and Penicillin Resistant (1633-2) Strains of *Staphylococcus aureus* in Presence and Absence of Serum Using a 10^4 Inoculum Dilution.

	MIC in μg/ml			
	Smith strain		1633-2 strain	
Penicillin	Without serum	With serum	Without serum	With serum
Phenoxymethyl	.04	.16	6.25	25.0
α-Phenoxyethyl	.08	.16	1.6	6.25
α-Phenoxypropyl	.08	.62	.8	6.25
α-Phenoxyisopropyl	.3	1.2	1.6	25.0
α-Phenoxybutyl	.6	2.5	6.25	50.0
α-Phenoxyisobutyl	.3	1.2	.8	12.5
Controls:				
Benzylpenicillin	.04	.08	12.5	25.0
Dimethoxyphenyl penicillin	1.2	2.5	1.6	1.6

its antibacterial activity. This compound had the lowest MIC against the 1633-2 strain in the presence of 50% serum.

It has been said that determination of the antistaphylococcal action of penicillins in the presence of low numbers of organisms is not realistic(7,8). Human staphylococcal abscesses are purported to have very high bacterial count. In Table III, the influence of inoculum size on the MIC of the resistant *S. aureus*, 1633-2 strain, is shown. Of the various penicillins in this series, only α-phenoxy-isobutyl penicillin is still inhibitory at the higher inoculum levels. There is a marked increase in MIC, however, as the inoculum dilution is varied from 10^5 to 10^2. This contrasts markedly with the MIC of the dimethoxyphenyl penicillin control which increases only slightly if at all at the highest inoculum levels.

TABLE III. Effect of Inoculum Size of a Penicillinase-Producing *S. aureus* Strain on Minimum Inhibitory Concentration of α-Phenoxyalkyl Penicillins.

	MIC in μg/ml			
	Inoculum dilution			
Penicillin	10^2	10^3	10^4	10^5
Phenoxymethyl	>100	>100	6.2	.4
α-Phenoxyethyl	>100	100	1.6	.8
α-Phenoxypropyl	>100	25	.8	.8
α-Phenoxyisopropyl	>100	12.5	1.6	.8
α-Phenoxybutyl	>100	100	6.2	3.12
α-Phenoxyisobutyl	12.5	1.6	.8	.8
Controls:				
Benzylpenicillin	>100	>100	12.5	.8
Dimethoxyphenyl penicillin	3.2	3.2	1.6	1.6

Since the effect of inoculum level is thought to be due to ability of the penicillin to withstand destruction by penicillinase, the relative rates of decomposition by staphylococcal penicillinase were determined for penicillin G, α-phenoxyisobutyl penicillin, and dimethoxyphenyl penicillin using the manometric technics described above. Expressing the results in terms of benzylpenicillin (equal to 100), α-phenoxyisobutyl penicillin was 20 and dimethoxyphenyl penicillin was 0.6. Thus, the α-phenoxyisobutyl penicillin was 5 times more resistant to destruction than penicillin G and dimethoxyphenyl penicillin was approximately 30 times more resistant than α-phenoxyisobutyl penicillin.

TABLE IV. Comparison of Intramuscular CD_{50} Values of Different Penicillins Using Penicillin Sensitive (Smith) and Penicillin Resistant (1633-2) Strains of *Staphylococcus aureus*.

	CD_{50} in mg/kg	
Penicillin	Smith strain	1633-2 strain
Phenoxymethyl	.8	>500
α-Phenoxyethyl	.5	>500
α-Phenoxypropyl	1.1	>500
α-Phenoxyisopropyl	3.7	>500
α-Phenoxybutyl	4.0	>500
α-Phenoxyisobutyl	6.0	170–240
Controls:		
Benzylpenicillin	1.1	>500
Dimethoxyphenyl penicillin	9.0	28–45

In vivo *experiments*. Results of mouse protection tests are reported in Table IV. All α-phenoxyalkyl penicillins studied protect mice against infection with the sensitive *S. aureus* strain at rather low doses, the lower homologs being most active. In the case of the penicillin-resistant strain, however, only α-phenoxyisobutyl penicillin showed some protection under the conditions of this test. The CD_{50} values are, however, considerably higher than those obtained with dimethoxyphenyl penicillin. Approximately 5 times more α-phenoxyisobutyl penicillin is required

than of dimethoxyphenyl penicillin to give the same degree of protection in these tests.

Discussion. It is interesting that as the side chain of the α-phenoxyalkyl penicillins becomes larger, activity against the penicillin-sensitive strain of *S. aureus* decreases. At the same time, in the case of some of the members of the series, there is an increase in activity against a penicillinase-producing *S. aureus*. This is especially seen with the compound α-phenoxyisobutyl penicillin. Of the members in the series examined, this compound shows the greatest *in vitro* and *in vivo* activity against a penicillinase-producing resistant staphylococcus. However, the activity is considerably less than that seen with dimethoxyphenyl penicillin. In the cases examined, the activity against the resistant staphylococcus paralleled the penicillinase resistance and it would thus appear that such compounds are active against these organisms by virtue of their ability to withstand degradation by the staphylococcal penicillinase.

The penicillins in this series have an asymmetric carbon in the side chain and therefore exist as diastereoisomeric mixtures. The method of preparation was such that approximately equal amounts of each diastereoisomer would be present. The data presented therefore represent the combined activities of both diastereoisomers.

The authors wish to thank Dr. L. C. Cheney and other members of the Organic Chemistry Dept., Bristol Laboratories, for making available the various synthetic penicillins.

1. Gourevitch, A., Hunt, G. A., Lein, J., *Antibiot. and Chemother.*, 1960, v10, 121.

2. Perron, Y. G., Minor, W. F., Holdrege, C. T., Gottstein, W. J., Godfrey, J. C., Crast, L. B., Babel, R. B., Cheney, L. C., *J. Am. Chem. Soc.*, 1960, v82, 3934.

3. Gourevitch, A., Hunt, G. A., Pursiano, T. A., Moses, A. J., Lein, J., A Symposium on the New Dimethoxyphenyl Penicillin. State University of New York, Upstate Med. Center, in press.

4. Hunt, G. A., Gourevitch, A., Lein, J., *J. Bact.*, 1958, v76, 453.

5. Henry, R. J., Housewright, R. D., *J. Biol. Chem.*, 1947, v167, 559.

6. Gilson, B. St.C., Parker, R. F., *J. Bact.*, 1948, v55, 801.

7. Geronimus, L. H., *New Eng. J. Med.*, 1960, v263, 349.

8. ———, *Bact. Proc.*, 1960, 103.

Received May 1, 1961. P.S.E.B.M., 1961, v107.

Studies on Glucose Metabolism in Cartilage *in vitro*.* (26656)

DANIEL S. BERNSTEIN, BERNARD LEBOEUF† AND GEORGE F. CAHILL, JR.‡
(Introduced by George W. Thorn)
Dept. of Medicine, Harvard Medical School and Peter Bent Brigham Hospital; Baker Clinic Research Laboratories, New England Deaconess Hospital and Dept. of Nutrition, Harvard School of Public Health, Boston, Mass.

Survey of the literature reveals a paucity of *in vitro* metabolic studies on carbohydrate metabolism in cartilaginous tissue, particularly in the presence of added hormones. However, the *in vivo* effects of growth hormone and insulin on mucopolysaccharide metabolism have been intensively investigated. Dorfman and Schiller(1) reported decreased uptake of S^{35} from injected $Na_2S^{35}O_4$ and C^{14} from acetate-1-C^{14} or glucose-U-C^{14} into chondroitin sulfate isolated from skin of diabetic rats. Insulin treatment returned these values toward normal. In hypophysectomized rats, there was a rapid decline in incorporation of these labeled compounds into chondroitin sulfate and hyaluronic acid. Growth hormone given to these hypophysectomized rats restored only incorporation into chondroitin sulfate without any apparent ef-

* Supported by grants from Arthritis and Rheumatism Foundation and from Nat. Inst. Health.

† Fellow of Life Insurance Medical Research Fund.

‡ Fellow of Metropolitan Foundation, Inc., Boston, Mass.

fect on incorporation into hyaluronic acid. Roden and Dorfman(2) found in experiments *in vivo* that glucose-6-C^{14} was equally distributed between the hexosamine and uronic acid moieties of mucopolysaccharides isolated from rat skin, and with the finding that the labeled carbon was still in the 6 position concluded that glucose is incorporated into mucopolysaccharide without splitting and reassembling of the carbon chain.

Since the rachitic weanling rat develops tibial epiphyseal plates approximately one-half centimeter thick containing a plentiful population of chondrocytes, this tissue was selected as suitable for *in vitro* experiments. In addition, the ease in preparing these animals and the rapidity whereby tissues can be excised, sliced and incubated are further factors contributing to the adequacy of this tissue for the experiments proposed.

Experimental. Male weanling rats of the Charles River strain (Sprague-Dawley descendants) were fed a high calcium rachitogenic diet (Nutritional Biochemicals Rachitogenic Diet #2, U.S.P.). For each experiment, 24 of these animals were killed after 3 to 4 weeks on the diet at which time gross examination revealed florid rickets. The hind legs were severed and all soft tissue rapidly dissected from the knee joint. Using the tibia as support, all adventitious tissue was scraped away from the epiphyses; the femur and its epiphysis were removed; the tibial epiphysis was sliced 4 to 5 times longitudinally with a razor blade and then transversely between the epiphysis and tibial shaft. From each epiphysis approximately 4-6 slices, each about ½ mm thick, were therefore prepared and randomly distributed into 6 piles on an iced glass plate until all animals had been killed. The 6 random piles each contained 175-250 mg of cartilage slices, and each pile was then placed in a 50 ml incubating flask containing 3 ml Krebs-Ringer bicarbonate buffer with 5 mM specifically labeled glucose-C^{14}. In different experiments, the order of the flasks was reversed to eliminate experimental selection. The flasks were then sealed with serum stoppers, placed on a Dubnoff apparatus and shaken 100 oscillations/minute for 3 hours at 38°C. For the first 10 minutes, the flasks were flushed with 95% O_2 : 5% CO_2 to re-equilibrate the buffer at pH 7.4.

At the end of incubation, the slices were removed and frozen for later analysis of polysaccharide content and radioactivity. To the medium was added 2 ml of 2 N H_2SO_4 and the liberated CO_2 trapped in 0.5 ml 1 N NaOH, converted to $BaCO_3$, plated on stainless steel planchets, weighed and counted in a thin window proportional flow counter(3, 4). Initial glucose specific activity was determined by preparation of the phenylosazone, recrystallization and counting as above.

Tissue mucopolysaccharide was isolated by a modification of Astrup's procedure where the frozen cartilage was pulverized in a Micro-Waring blendor into a smooth watery homogenate(5,6). This was diluted to 50 ml with distilled water and 25 ml of saturated benzidine hydrochloride added. After stirring, 2 ml of 5 N HCl was added, the mixture centrifuged and the supernatant fluid discarded. To the precipitate was added 10 ml of 10% NH_3 and 50 ml of ether, and the mixture transfererd to a separatory funnel. The aqueous layer was filtered and the filter paper rinsed with 10% NH_3. The filtrate was collected and solid NaCl added to a concentration of approximately 0.5%. Four volumes of absolute ethanol were added, the solution cooled and the white flocculent precipitate centrifuged. The precipitate was dissolved in 10 to 15 ml of water and dialyzed in a Visking 24/40 membrane against 20 to 30 volumes of water overnight at 4°C. It was then lyophilized, the residue weighed and dissolved in distilled water. An aliquot was dried on a steel planchet, weighed, and counted.

Glucose-1-C^{14} and glucose-6-C^{14} were purchased from New England Nuclear Corp. Crystalline insulin was kindly provided by Dr. W. R. Kirtley of Eli Lilly and Co. and bovine growth hormone, lot #R50109, was obtained through the courtesy of the Endocrinology Study Section of Nat. Inst. of Health.

Results. In Table I are summarized the results of 7 experiments comparing the metabolism of glucose-1-C^{14} and glucose-6-C^{14}

TABLE I. Effect of Insulin and Growth Hormone on the Oxidation of Glucose-1-C^{14} or Glucose-6-C^{14} to CO_2 in Rachitic Rat Cartilage *In Vitro*. Glucose, 5 mM; insulin, 0.1 unit/ml; growth hormone, 0.33 mg/ml. Values in μmoles of specifically-labeled glucose carbon recovered in total medium and gaseous CO_2 per g wet cartilage per 3 hr incubation. Each experiment contains pooled tissues from 24 animals with stand. errors of the mean.

	Control		Insulin		Growth hormone	
Exp.	C-1	C-6	C-1	C-6	C-1	C-6
1	1.54	.88	3.53	1.35	1.75	.98
2	2.81	1.40	3.90	1.59	2.80	1.40
3	2.12	1.43	4.04	1.54	2.25	.88
4	2.60	1.23	—	—	2.24	1.16
5	1.98	1.01	2.63	1.16	2.22	1.08
6	2.98	1.40	3.58	1.53	2.63	1.61
7	2.18	1.10	3.59	1.37	2.62	1.60
Mean ± S.E. of mean	2.32 ± .19	1.21 ± .08	3.52 ± .20	1.42 ± .06	2.33 ± .13	1.24 ± .11

to CO_2 in control tissues and in tissues incubated with growth hormone. In 6 of these experiments, the effects of insulin were also studied. From the results it is clear that in both control and hormone treated tissues there is a significantly greater oxidation of glucose carbon-1 compared to carbon-6, suggesting operation of the direct oxidative pathway. Insulin significantly increases oxidation of both carbon-1 ($P<0.01$) and carbon-6 ($P<0.01$) to CO_2, but there was no apparent alteration in presence of the growth hormone preparation.

Isolation of tissue polysaccharide and determination of specific activity resulted in an overall mean value of 15.3 $\mu\mu$M of specifically-labeled glucose incorporated into 1 mg of polysaccharide. In control tissues, mean glucose incorporation was 12.4 $\mu\mu$M/mg and in insulin treated tissues 16.1 $\mu\mu$M/mg polysaccharide. However, analysis of paired incubations showed an increase of 19 ± 12% in presence of insulin which was only suggestive of significance ($P<0.2$). On the other hand, mean polysaccharide incorporation in presence of growth hormone was 18.3 $\mu\mu$M/mg and paired data showed a more significant increase of 61 ± 19% ($P<0.05$) compared to the paired control tissues.

More surprising, mean incorporation of carbon-1 into polysaccharide was 18.2 $\mu\mu$M and that of carbon-6 12.1 $\mu\mu$M. Again, analysis of paired tissues showed this difference to be significant, namely a 60 ± 17% ($P<0.05$) greater incorporation of glucose carbon-1 compared to carbon-6. No significant alteration in this ratio was effected by either insulin or growth hormone.

Discussion. The data show several definite metabolic characteristics of cartilage cells derived from rachitic rats. One characteristic is a significantly greater oxidation of glucose carbon-1 compared to carbon-6, suggesting operation of the direct oxidative pathway. However, recovery of specifically-labeled carbon in tissue polysaccharide does not support this selective loss of carbon-1; in fact, more carbon-1 than carbon-6 is recovered in polysaccharide. This discrepancy cannot be resolved unless other pathways of asymmetric metabolism are operative, such as failure of triose isomerase effectively to equilibrate triose phosphates or another pathway, such as that involving formation of uridine diphosphoglucuronic acid and eventual cleavage of carbon-6 to CO_2 (the glucuronic acid pathway). That the latter pathway is operative in rachitic rat cartilage is given support by the recent isolation of uridine diphosphate and several of its glycosides, namely glucose, glucuronic acid and acetyl galactosamine (unpublished observations).

The stimulatory effects of the 2 hormones are clearly separable, insulin increases glucose oxidation to CO_2 with only a suggestive increase in polysaccharide synthesis whereas growth hormone does not increase glucose oxidation but significantly increases polysaccharide synthesis. Extrapolation of these results to *in vivo* metabolism is dangerous for many reasons including the extremely

unphysiological dose of hormones used in these experiments, the lack of homogeneity of the growth hormone preparation, and most important, the overall unphysiological milieu of *in vitro* incubation.

Summary. Cartilage slices obtained from weanling rachitic rats oxidize glucose carbon-1 to CO_2 more rapidly than carbon-6. Insulin increases oxidation of both carbons whereas growth hormone has no effect. Growth hormone significantly increases glucose carbon incorporation into tissue polysaccharide.

1. Dorfman, A., Schiller, S., *Recent Progress in Hormone Research,* 1958, v14, 427.

2. Roden, L., Dorfman, A., *J. Biol. Chem.,* 1958, v233, 1030.

3. Robinson, C. V., *Science,* 1950, v112, 198.

4. Karnovsky, M. L., Foster, J. M., Gidez, L. I., Hagerman, D. D., Robinson, C. V., Solomon, A. K., Villee, C. A., *Anal. Chem.,* 1955, v27, 852.

5. Astrup, P., *Acta Pharm. et Tox.,* 1947, v3, 165.

6. Bernstein, D. S., Handler, P., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 339.

Received March 24, 1961. P.S.E.B.M., 1961, v107.